Automated DNA Sequencing

Chemistry Guide

Applied Biosystems

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1 Introduction

New DNA Sequencing Chemistry Guide

Purpose

Since the original *DNA Sequencing Chemistry Guide* was published in early 1995, Applied Biosystems has released two new instrument platforms, five new sequencing chemistries, and a new sequencing enzyme.

To accommodate this new information, we have written the *Automated DNA Sequencing Chemistry Guide*. This updated guide provides the following:

- An introduction to automated DNA sequencing
- Descriptions of Applied Biosystems sequencing instruments, chemistries, and software
- Detailed protocols for preparing DNA templates, performing cycle sequencing, and preparing the extension products for electrophoresis
- Guidelines for optimizing electrophoresis and interpreting and troubleshooting sequencing data

Introduction to Automated DNA Sequencing

Sanger Dideoxy Sequencing

DNA polymerases copy single-stranded DNA templates, by adding nucleotides to a growing chain (extension product). Chain elongation occurs at the 3´ end of a primer, an oligonucleotide that anneals to the template. The deoxynucleotide added to the extension product is selected by base-pair matching to the template.

The extension product grows by the formation of a phosphodiester bridge between the 3´-hydroxyl group at the growing end of the primer and the 5´-phosphate group of the incoming deoxynucleotide (Watson *et al.*, 1987). The growth is in the 5´→3´ direction (Figure 1-1).

DNA polymerases can also incorporate analogues of nucleotide bases. The dideoxy method of DNA sequencing developed by Sanger *et al.* (1977) takes advantage of this ability by using 2´,3´-dideoxynucleotides as substrates. When a dideoxynucleotide is incorporated at the 3´ end of the growing chain, chain elongation is terminated selectively at A, C, G, or T because the chain lacks a 3´-hydroxyl group (Figure 1-1).

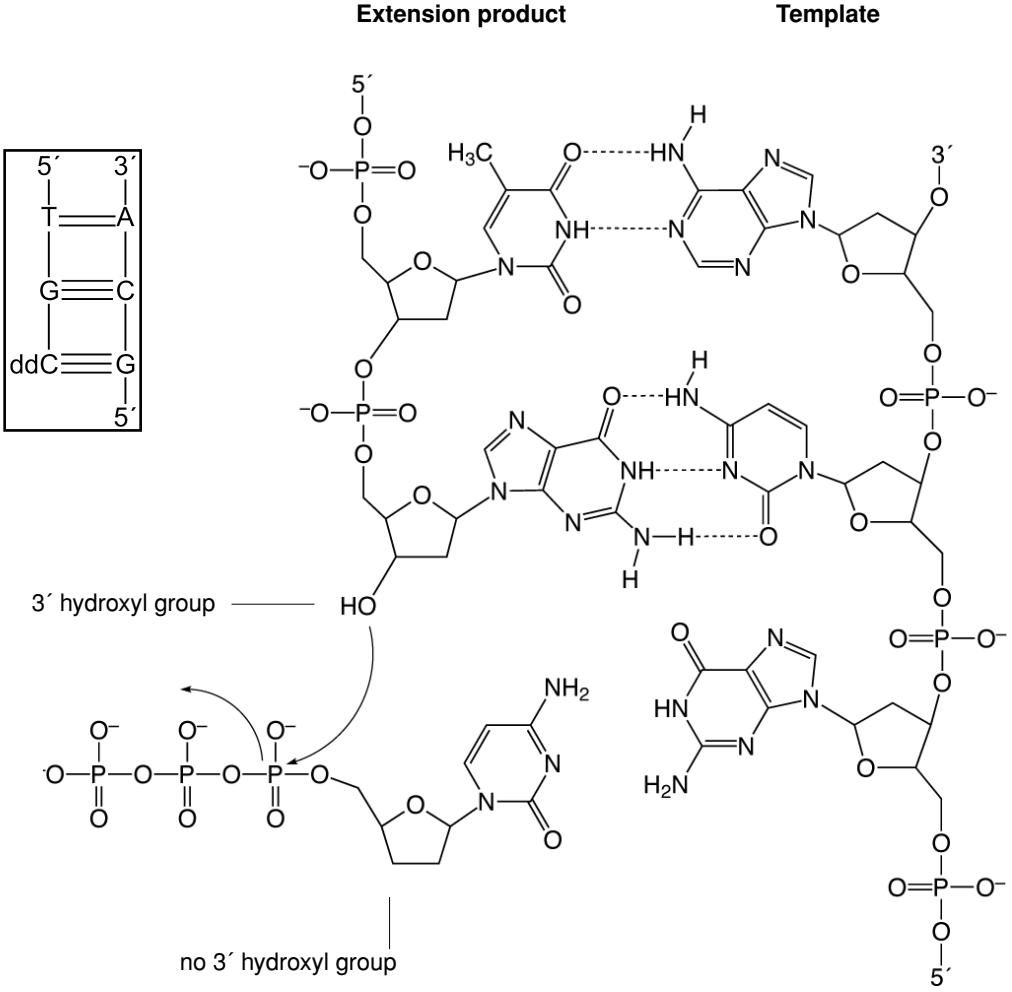

Figure 1-1 DNA strand synthesis by formation of phosphodiester bonds. The chain is terminated by the use of dideoxycytidine triphosphate (ddC) in place of deoxycytidine triphosphate (dCTP). The inset shows a schematic representation of the process.

Fluorescent Sequencing

In the Applied Biosystems strategy for automated fluorescent sequencing, fluorescent dye labels are incorporated into DNA extension products using 5´-dye labeled primers (dye primers) or 3´-dye labeled dideoxynucleotide triphosphates (dye terminators). The most appropriate labeling method to use depends on your sequencing objectives, the performance characteristics of each method, and on personal preference.

Applied Biosystems DNA sequencers detect fluorescence from four different dyes that are used to identify the A, C, G, and T extension reactions. Each dye emits light at a different wavelength when excited by an argon ion laser. All four colors and therefore all four bases can be detected and distinguished in a single gel lane or capillary injection (Figure 1-2).

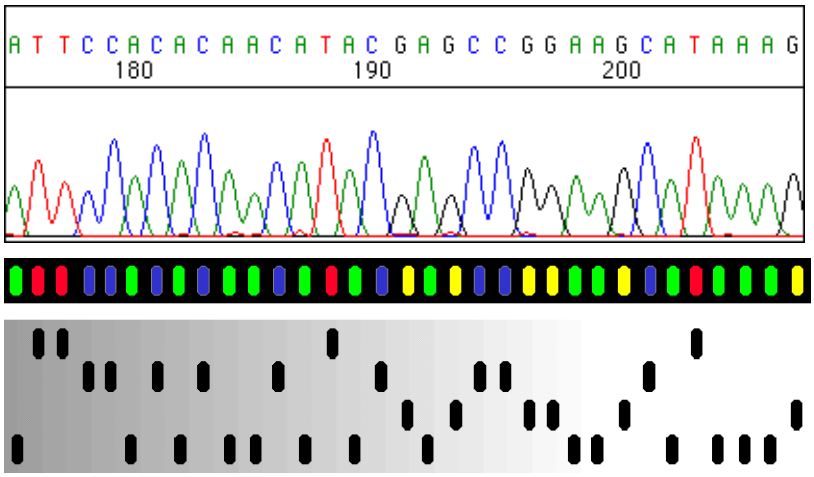

Figure 1-2 Four-color/one-lane fluorescent sequencing vs. one-color/four-lane method such as radioactive sequencing

Cycle Sequencing

Cycle sequencing is a simple method in which successive rounds of denaturation, annealing, and extension in a thermal cycler result in linear amplification of extension products (Figure 1-3). The products are then loaded onto a gel or injected into a capillary. All current ABI PRISM DNA sequencing kits use cycle sequencing protocols.

See Chapter 3 for information on cycle sequencing protocols.

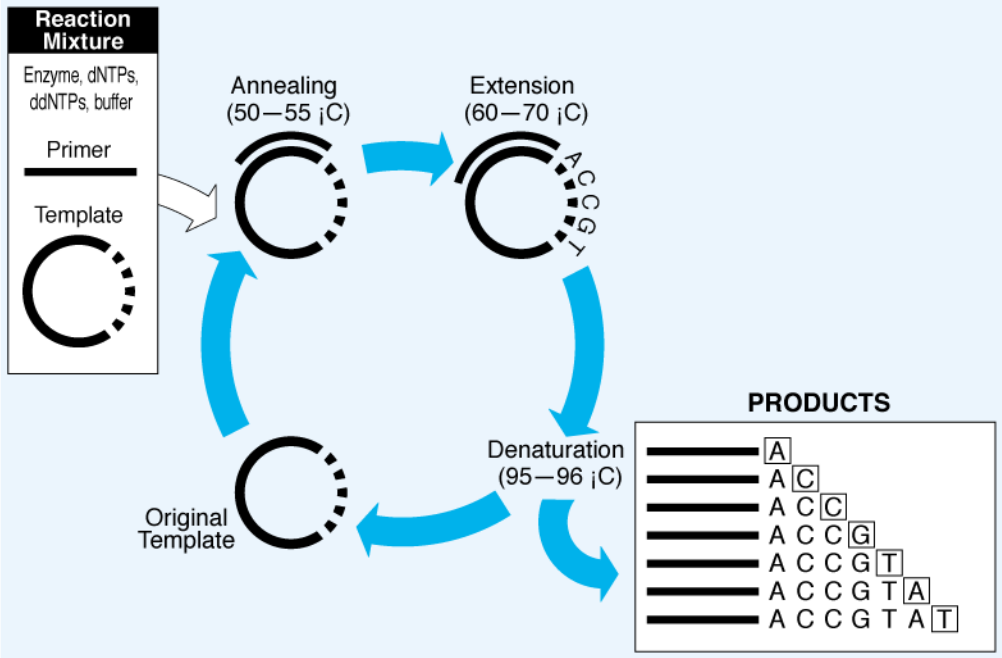

Figure 1-3 Cycle sequencing

Advantages of Cycle Sequencing

- Protocols are robust and easy to perform.
- Cycle sequencing requires much less template DNA than single-temperature extension methods.
- Cycle sequencing is more convenient than traditional single-temperature labeling methods that require a chemical denaturation step for double-stranded templates.
- High temperatures reduce secondary structure, allowing for more complete extension.
- High temperatures reduce secondary primer-to-template annealing.
- The same protocol is used for double- and single-stranded DNA.
- The protocols work well for direct sequencing of PCR products (see page 3-14).
- Difficult templates, such as bacterial artificial chromosomes (BACs), can be sequenced.

ABI PRISM Sequencing Chemistries

AmpliTaq DNA Polymerase, FS

AmpliTaq® DNA Polymerase, FS is the sequencing enzyme used in ABI PRISM cycle sequencing kits. It is a mutant form of *Thermus aquaticus* (Taq) DNA polymerase and contains a point mutation in the active site, replacing phenylalanine with tyrosine at residue 667 (F667Y). This mutation results in less discrimination against dideoxynucleotides, and leads to a much more even peak intensity pattern (Tabor and Richardson, 1995).

AmpliTaq DNA Polymerase, FS also contains a point mutation in the amino terminal domain, replacing glycine with aspartate at residue 46 (G46D), which removes almost all of the 5´→3´ nuclease activity. This eliminates artifacts that arise from the exonuclease activity.

The enzyme has been formulated with a thermally stable inorganic pyrophosphatase that cleaves the inorganic pyrophosphate (PP_i) byproduct of the extension reaction and prevents its accumulation in the sequencing reaction.

In the presence of high concentrations of PP_i the polymerization reaction can be reversed (Kornberg and Baker, 1992), a reaction called pyrophosphorolysis. In this reaction, a nucleoside monophosphate is removed from the extension product with the addition of PP_i to form the nucleoside triphsphate.

In a sequencing reaction, if a dideoxynucleotide is frequently removed at a particular position and replaced by a deoxynucleotide, eventually there is little or no chain termination at that location. This results in a weak or missing peak in the sequence data (Tabor and Richardson, 1990).

Dye-Labeled Terminators

With dye terminator labeling, each of the four dideoxy terminators (ddNTPs) is tagged with a different fluorescent dye. The growing chain is simultaneously terminated and labeled with the dye that corresponds to that base (Figure 1-4).

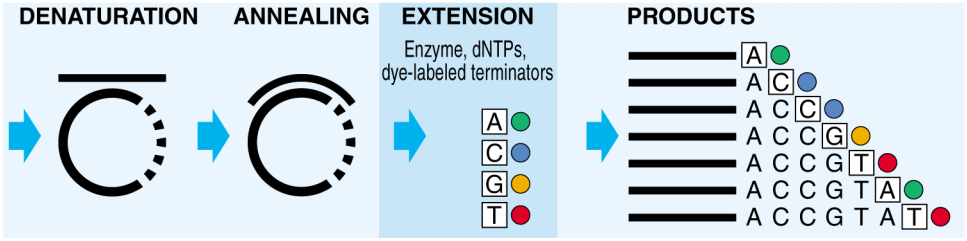

Figure 1-4 One cycle of dye terminator cycle sequencing

Features of Dye-labeled Terminator Reactions

- An unlabeled primer can be used.
- Dye terminator reactions are performed in a single tube. They require fewer pipetting steps than dye primer reactions.
- Four-color dye labeled reactions are loaded in a single gel lane or capillary injection.
- False stops, *i.e.*, fragments that are not terminated by a dideoxynucleotide (see page 7-30), go undetected because no dye is attached.

See Chapter 2 for information on ABI PRISM™ DNA sequencing kits.

Dye-Labeled Primers

With dye primer labeling, primers are tagged with four different fluorescent dyes. Labeled products are generated in four separate base-specific reactions. The products from these four reactions are then combined and loaded into a single gel lane or capillary injection (Figure 1-5).

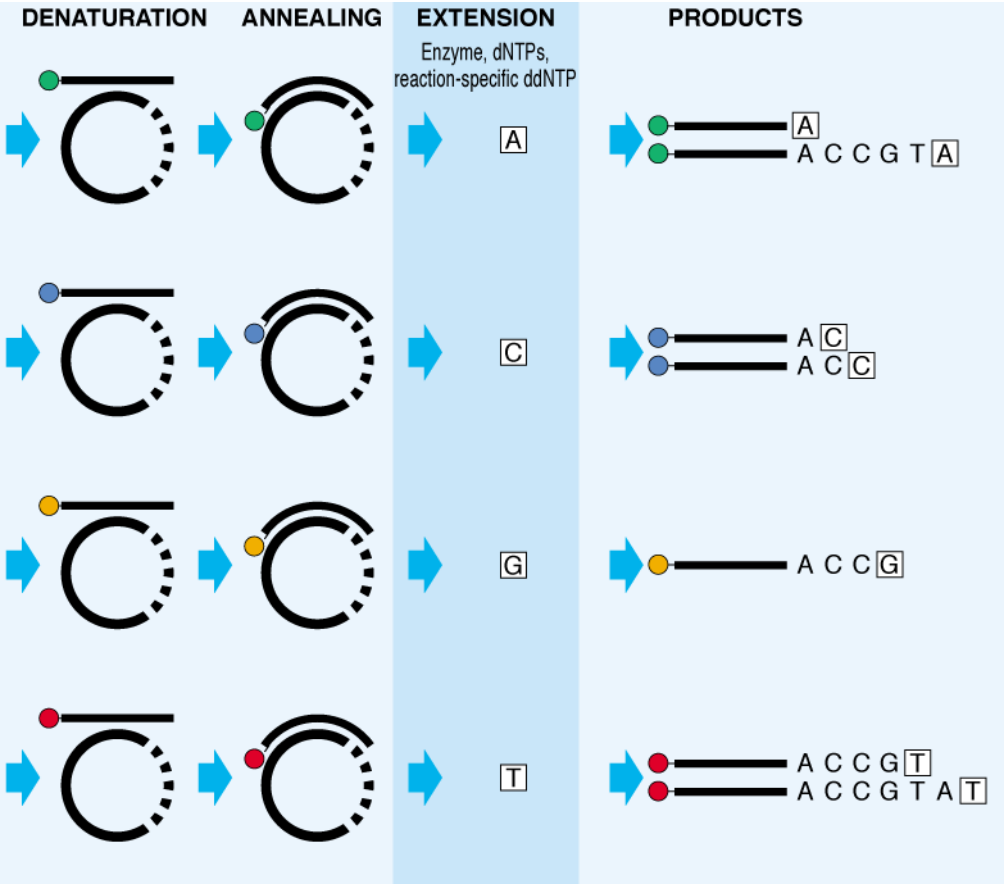

Figure 1-5 One cycle of dye primer cycle sequencing

Features of Dye-labeled Primer Reactions

- Dye primer chemistries generally produce more even signal intensities than dye terminator chemistries.
- Labeled primers are available for common priming sites. Custom primers can also be labeled.
- Four-color dye-labeled reactions are loaded onto a single lane or capillary injection.

See Chapter 2 for information on ABI PRISM™ DNA sequencing kits.

Applied Biosystems DNA Sequencing Instruments

ABI 373 DNA Sequencer

The ABI™ 373 DNA Sequencer is an automated instrument for analyzing fluorescently labeled DNA fragments by gel electrophoresis. You can use three sizes of gel plates for sequencing applications: 24-cm, 34-cm and 48-cm well-to-read lengths (see Table 1-1 on page 1-10). The longer the well-to-read length, the better the resolution of the gel.

Sequencing reaction products labeled with four different fluorescent dyes are loaded into each lane of a 0.3-mm or 0.4-mm vertical slab gel made of polymerized acrylamide or acrylamide derivatives. You can run up to 36 lanes simultaneously on a single gel.

The dye-labeled DNA fragments migrate through the acrylamide gel and separate according to size. At the lower portion of the gel they pass through a region where a laser beam scans continuously across the gel. The laser excites the fluorescent dyes attached to the fragments, and they emit light at a specific wavelength for each dye.

The fluorescence intensity is detected by a photomultiplier tube (PMT) and recorded as a function of time. A moving stage contains the optical equipment (filter wheel and photomultiplier tube). The PMT detects the fluorescence emission and converts it into a digital signal. Each time the stage traverses across the gel (a scan) a different bandpass filter is positioned in front of the PMT to detect each of the four dyes.

A single scan of the gel with one filter takes 1.5 seconds and measures signal in 194 channels. A complete scan with four filters takes 6 seconds and equals one data point. The data is then transmitted to the Macintosh® computer and stored for processing. The Sequencing Analysis software (see page 1-16) interprets the result, calling the bases from the fluorescence intensity at each data point.

Refer to the *373 DNA Sequencing System User's Manual* (P/N 902376) for more information.

XL Upgrade

The ABI 373 DNA Sequencer with XL Upgrade increases the number of samples that can be analyzed simultaneously. This increased throughput is made possible by reengineering the instrument to collect data from 388 channels instead of 194. With the XL Upgrade, the operation of the ABI 373 DNA Sequencer is controlled from the Power Macintosh® computer supplied with the upgrade.

After the initial calibration by the Field Service Engineer, the instrument automatically increases the PMT voltage to compensate for the smaller amount of signal generated per lane when running 48- or 64-lane gels.

The XL Upgrade also includes new combs and spacers. For sequencing applications, 48-well and 64-well shark' s tooth combs are available. You can still use 24-well or 36-well combs if desired.

Note These combs are not interchangeable with combs for the ABI PRISM® 377 DNA Sequencer.

Refer to the *373 DNA Sequencer With XL Upgrade User's Manual* (P/N 904258) for more information.

Filter Sets

The ABI 373 and ABI 373 with XL Upgrade DNA Sequencers use filters mounted on a filter wheel to separate light of different wavelengths. The instruments record the light intensity in four regions, collectively called Filter Set A, centered at the following wavelengths:

- Four-filter wheel: 540 nm, 560 nm, 580 nm, 610 nm
- Five-filter wheel: 531 nm, 560 nm, 580 nm, and 610 nm

Note The five-filter wheel instruments also have Filter Set B (531 nm, 545 nm, 560nm, and 580 nm), but it is not used with existing Applied Biosystems sequencing chemistries. Filter Set B was used for the T7 (Sequenase) terminator chemistries, which have been discontinued.

BigDye Filter Wheel

To use the new dRhodamine terminator, BigDye™ terminator, and BigDye™ primer sequencing chemistries (see Chapter 2) on the ABI 373 and ABI 373 with XL Upgrade DNA Sequencers, the ABI PRISM™ BigDye™ Filter Wheel has been developed.

Its Filter Set A is as follows: 540 nm, 570 nm, 595 nm, and 625 nm.

Note The BigDye Filter Wheel also has Filter Set B (540 nm, 555 nm, 570, and 595 nm), but it is not used with existing Applied Biosystems sequencing chemistries.

Refer to the *Using the ABI 373 BigDye Filter Wheel User Bulletin* (P/N 4304367) for more information.

ABI PRISM 377 DNA Sequencer

The ABI PRISM 377 DNA Sequencer is a medium- to high-throughput, automated instrument for analyzing fluorescently labeled DNA fragments by gel electrophoresis. You can use two sizes of gel plates for sequencing applications: 36-cm and 48-cm well-to-read lengths. The 48-cm well-to-read plates are used to obtain longer read lengths.

Sequencing reaction products labeled with four different fluorescent dyes are loaded into each lane of a 0.2-mm vertical slab gel made of polymerized acrylamide or acrylamide derivatives. You can run up to 36 lanes simultaneously on one gel.

The dye-labeled DNA fragments migrate through the acrylamide gel and separate according to size. At the lower portion of the gel they pass through a region where a laser beam scans continuously across the gel. The laser excites the fluorescent dyes attached to the fragments, and they emit light at a specific wavelength for each dye.

The light is collected in 194 channels during each scan and separated according to wavelength by a spectrograph onto a cooled, charge-coupled device (CCD) camera, so all four types of fluorescent emissions can be detected with one pass of the laser. The data collection software collects the light intensities from the CCD at particular wavelength bands (virtual filters) and stores them on a Power Macintosh computer as digital signals for processing. The Sequencing Analysis software (see page 1-16) interprets the result, calling the bases from the fluorescence intensity at each data point.

Refer to the ABI PRISM 377 *DNA Sequencer User's Manual* (P/N 903433) for more information.

377-18

The ABI PRISM 377-18 DNA Sequencer is a lower-cost, lower-throughput version of the ABI PRISM 377 DNA Sequencer. It can run up to 18 lanes on a single gel.

XL Upgrade

The ABI PRISM 377 DNA Sequencer with XL Upgrade increases the number of samples that can be analyzed simultaneously. This increased throughput is made possible by reengineering the instrument to collect data from 388 channels instead of 194 during each scan.

The XL Upgrade also includes new combs. For sequencing applications, 48-well and 64-well shark' s tooth combs are available. You can still use 36-well or other lower lane density combs if desired.

Refer to the ABI PRISM 377 *DNA Sequencer XL Upgrade User's Manual* (P/N 904412) for more information.

96-Lane Upgrade

The ABI PRISM 377 DNA Sequencer with 96-Lane Upgrade increases the number of samples that can be run on each gel. The increased throughput is made possible by reengineering the instrument to collect data from 480 channels instead of 388 for the ABI PRISM 377 DNA Sequencer with XL Upgrade or 194 for the ABI PRISM 377 DNA Sequencer.

The 96-lane upgrade includes new combs and new notched front glass plates. You can still use lower lane density combs, but only with the original notched front glass plates that were provided with the instrument.

The new notched front glass plate has a bevel in the loading region that increases the thickness of the gel in this region from 0.2 mm to 0.4 mm. In addition, the scan region has been increased from 6 inches to 7.5 inches. This makes sample loading easier than for a 64-lane gel.

Refer to the ABI PRISM 377 *DNA Sequencer 96-Lane Upgrade User's Manual* (P/N 4305423) for more information.

Gel Electrophoresis Instruments

Table 1-1 Applied Biosystems Gel Electrophoresis Instruments

Instrument	Well-to-Read Length (cm)	Number of Lanes	Maximum Throughput (bases/hr)[a]	Detection System	Computer
ABI 370	24	16	800	PMT, 4-filter wheel	HP Vectra
ABI 373		24	1200		Macintosh
ABI 373 Leon Model	6, 12, 24, 34	24, 36	1800	PMT, 5-filter wheel	
ABI 373 Stretch Model	6, 12, 24, 34, 48				
ABI 373 with XL Upgrade	24 or 6, 12, 24, 34 or 6, 12, 24, 34, 48	24, 36, 48, 64	3200		Power Macintosh
ABI 373 with BigDye Filter Wheel[b]		24, 36 or 24, 36, 48, 64	1800 or 3200	PMT, new 5-filter wheel	Macintosh or Power Macintosh
ABI PRISM 377	12, 36, 48	24, 36	7200	CCD camera, spectrograph	Power Macintosh
ABI PRISM 377-18		18	3600		
ABI PRISM 377 with XL Upgrade		24, 36, 48, 64	12,800		
ABI PRISM 377 with 96-Lane Upgrade		24, 36, 48, 64, 96	19,200		

a. Maximum throughput = maximum number of lanes × maximum electrophoresis speed (50 bph for ABI 370 and ABI 373 models, 200 bph for ABI PRISM 377 models)

b. Allows use of dRhodamine-based chemistries on any ABI 373 or ABI 373 with XL Upgrade instrument with a 5-filter wheel. See page 1-8 for ABI 373 filter sets.

ABI PRISM 310 Genetic Analyzer

The ABI PRISM® 310 Genetic Analyzer is an automated instrument for analyzing fluorescently labeled DNA fragments by capillary electrophoresis.

The sequencing reaction sample tubes are placed in an autosampler tray that holds either 48 or 96 samples. The autosampler successively brings each sample into contact with the cathode electrode and one end of a glass capillary filled with a separation polymer. An anode electrode at the other end of the capillary is immersed in buffer.

The sample enters the capillary as current flows from the cathode to the anode. The short period of electrophoresis conducted while the capillary and cathode are immersed in the sample is called electrokinetic injection. The sample forms a tight band in the capillary during this injection. The end of the capillary near the cathode is then placed in buffer. Current is applied again to continue electrophoresis.

When the DNA fragments reach a detector window in the capillary, a laser excites the fluorescent dye labels. Emitted fluorescence from the dyes is collected once per second by a cooled, charge-coupled device (CCD) camera at particular wavelength bands (virtual filters) and stored as digital signals on a Power Macintosh computer for processing. The Sequencing Analysis software (see page 1-16) interprets the result, calling the bases from the fluorescence intensity at each data point.

Refer to the ABI PRISM 310 *Genetic Analyzer User's Manual* (P/N 903565) for more information.

Virtual Filter Sets

ABI PRISM 310 and ABI PRISM 377 (All Models)[1]

These instruments use virtual filter sets to detect light intensity in four non-overlapping regions on a CCD camera. Each region corresponds to a wavelength range that contains or is close to the emission maximum of an ABI PRISM dye.

The process is similar to using a physical filter to separate light of different wavelengths. However, the filter sets are called "virtual filters" because the instruments use no physical filtering hardware to perform the separation.[2]

The exact positions of the CCD regions and the dye combinations appropriate to these positions depend upon the virtual filter set used. For example, with Virtual Filter Set E the instrument records the light intensity in four regions, or "windows," centered at 540 nm, 570 nm, 595 nm, and 625 nm. The window positions in each virtual filter set have been optimized to provide the maximum possible separation among the centers of detection for the different dyes while maintaining good signal strength.

The Data Collection Software color-codes the intensity displays from the four light-collection regions. These appear as the blue, green, black (yellow on gel images), and red peaks in the raw data.

The Sequencing Analysis Software uses the same four colors to color-code analyzed data from all dye/virtual filter set combinations. The display colors represent the relative, not the actual, detection wavelengths. For consistency, the software always displays analyzed data with A as green, C as blue, G as black, and T as red in the electropherogram view.

Table 1-2 shows the wavelengths of the "windows" in the virtual filter sets used in cycle sequencing applications.

Table 1-2 Wavelength Ranges of Virtual Filter Sets

Virtual Filter Set	Color	Wavelength Range of Virtual Filter (nm)
A	blue	530–541
	green	554–564
	yellow/black	581–591
	red	610–620
E	blue	535–545
	green	565–575
	yellow/black	590–600
	red	620–630

1. Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.
2. The ABI PRISM 310 Genetic Analyzer and ABI PRISM 377 DNA Sequencer have a long-pass filter to prevent light from the instrument's argon ion laser from interfering with the detection of the dye signals.

Data Collection and Analysis Settings

Overview

This section is intended to provide an introduction to the data collection and analysis settings, which are dealt with in more detail in Chapter 6.

Many users sequence DNA using more than one chemistry. Take care when entering data collection and analysis settings in the software. If your data is analyzed with the wrong software settings, the resulting electropherograms will show overlapping peaks and gaps between peaks rather than the evenly spaced peaks characteristic of correctly analyzed data.

Run Modules

ABI 373 with XL Upgrade

A run module file contains all the parameters required for a particular function or application. The parameters include the following:

- Electrophoresis power
- Current and voltage settings
- Laser settings
- Scanner settings
- PMT settings

There are three types of run module files. Not all of the parameters listed above are in each module file.

- Plate Check

  This module is for checking the cleanliness and alignment of the gel plates. Laser, scanning, and PMT settings are associated with it.

- Pre Run

  This module is for prerunning sequencing gels. Laser, scanning, electrophoresis, and PMT settings are associated with it.

- Seq Run

  This module is for running sequencing gels. Laser, scanning, electrophoresis, and PMT settings are associated with it.

IMPORTANT When you select a run module, the filter set is chosen automatically. You must edit the run module to change the filter set used to collect the data. Refer to the *373 DNA Sequencer With XL Upgrade User's Manual* (P/N 904258) for more information.

Note The ABI 373 DNA Sequencer does not use run modules. Run parameters are set on the instrument's keypad. Refer to the *373 DNA Sequencing System User's Manual* (P/N 902376) for information on setting run parameters.

ABI PRISM 310 and ABI PRISM 377 (All Models)

A run module file contains all the parameters required for a particular function or application. The parameters include the following:

- Electrophoresis voltage
- Current and power settings
- Laser settings
- Scanner settings (ABI PRISM 377 DNA Sequencer only)

- Virtual filters and CCD gain and offset
- Run temperature settings
- Injection time and voltage (ABI PRISM 310 Genetic Analyzer)

There are three types of module files. Not all of the parameters listed above are in each module file.

- Plate check

  These modules are for checking the cleanliness and alignment of the gel plates. Laser, scanning, virtual filter, and CCD conditions are associated with these types of files.

- Prerun

  These modules are for prerunning the gel or polymer. Laser, scanning, virtual filter, and electrophoresis, CCD, and gel temperature conditions are associated with these types of files.

Note Plate check and prerun modules are not used with the ABI PRISM 310 Genetic Analyzer.

- Run

  These modules are for running the gel or polymer. Laser, scanning, virtual filter, CCD, and electrophoresis parameters and gel temperature are associated with these types of files.

IMPORTANT When you select a run module, the virtual filter set is chosen automatically. You must be careful to select the correct run module for your sequencing chemistry.

The available run modules are listed in Table 6-1 on page 6-2.

Dye Set/Primer Files

Mobility Correction

The different dyes affect the electrophoretic mobility of cycle sequencing extension products. The relative mobility of the dye-labeled fragments is specific to each sequencing chemistry (see page 6-4 for more information). Under the same set of conditions, the mobilities are very reproducible.

The analysis software is able to compensate for these mobility differences by applying mobility shifts to the data so that evenly spaced peaks are presented in the analyzed data. The files that contain the mobility shift information are called dye set/primer files.

Dye set/primer files also tell the Sequencing Analysis software (see page 1-16) the following:

- Which matrix file in the instrument file (see page 1-14) to use to analyze the data
- Dye/base relationships for converting raw data colors to base calls (see page 2-14)

The dye set/primer files available are listed in Table 6-2 on page 6-5.

Multicomponent Analysis

Multicomponent analysis is the process that separates the four different fluorescent dye colors into distinct spectral components. Although each of these dyes emits its maximum fluorescence at a different wavelength, there is some overlap in the emission spectra between the four dyes (Figure 1-6). The goal of multicomponent analysis is to isolate the signal from each dye so that there is as little noise in the data as possible.

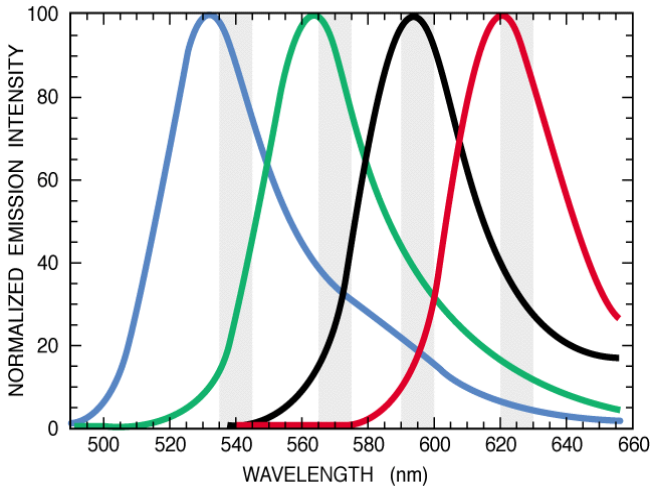

Figure 1-6 Spectral overlap of the dRhodamine dyes in the four virtual filters (vertical gray bars) of Filter Set E

The precise spectral overlap between the four dyes is measured by running DNA fragments labeled with each of the dyes in separate lanes of a gel or in separate injections on a capillary. These dye-labeled DNA fragments are called matrix standards.

The Data Utility software (see page 6-7) then analyzes the data from each of the four matrix standard samples and creates an instrument file. The instrument file contains three matrix files, which have tables of numbers with four columns and four rows (Figure 1-7 on page 1-15). These numbers are normalized fluorescence intensities and represent a mathematical description of the spectral overlap that is observed between the four dyes.

The rows in the tables represent the virtual filters and the columns represent the dyes. The top lefthand value, 1.000, represents the normalized fluorescence of the blue dye in the blue filter. It follows that all matrix tables should have values of 1.000 on the diagonal from top left to bottom right.

The other values in the table should all be less than 1. These values represent the amount of spectral overlap observed for each dye in each virtual filter. For example, the values in the third row reflect quantitatively the amount of each dye detected in the third (“yellow”) virtual filter.

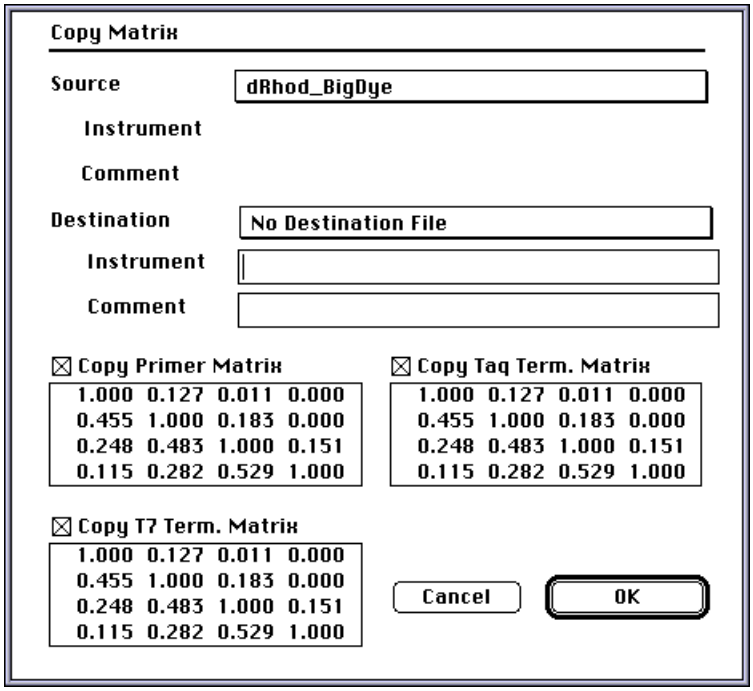

Figure 1-7 Instrument file created in the Data Utility software, indicating the values obtained with the dRhodamine matrix standards for Filter Set E on a particular ABI PRISM 377 instrument

Note that the numbers decrease moving away from the diagonal in any direction. For example, in the first column the amount of blue fluorescence seen through the red filter (fourth row) should be less than that seen in the yellow filter (third row), which should be less than that seen in the green filter (second row).

These values will vary between different instruments and between filter sets on a single instrument. An instrument file must be made for each filter set used on each instrument.

The instrument file is created for a specific filter set or virtual filter set when the instrument is installed. Whenever a new filter set is used, a new instrument file must be created for that filter set. Refer to your instrument user's manual or the protocol for the sequencing chemistry you are using for instructions on creating instrument files.

The appropriate instrument file can be applied to data on subsequent capillary runs or gels on the same instrument, as long as the same filter set is used. This is because the spectral overlap between the four dyes is very reproducible.

Multicomponent analysis of sequencing data is performed automatically by the Sequencing Analysis software (see below), which applies a mathematical matrix calculation, using the values in the instrument file, to all sample data.

See page 6-7 for instructions for creating instrument files.

What Is In a Matrix File

The matrix files in an instrument file are used for specific types of chemistry, and provide information to the Sequencing Analysis software to allow it to correct for spectral overlap.

Matrix files also contain the following:

- Baselining algorithm for the chemistry being used
- Information that the Sequencing Analysis software uses to determine Peak 1 Locations and Start Points for data analysis

Sequencing Analysis Software

The DNA Sequencing Analysis Software analyzes the raw data collected by the Data Collection software:

- Tracks gel files (if using the ABI 373 or ABI PRISM 377 DNA Sequencer)
- Extracts sample information from gel files (if using the ABI 373 or ABI PRISM 377 DNA Sequencer)
- Performs multicomponent analysis
- Applies mobility corrections
- Normalizes the base spacing
- Baselines data
- Determines analysis starting points
- Calls bases

See Chapter 7 for information on interpreting and troubleshooting sequencing data.

Refer to the *ABI PRISM DNA Sequencing Analysis Software User's Manual* for specific information about the Sequencing Analysis software.

ABI PRISM DNA Sequencing Chemistries 2

Overview

In This Chapter This chapter describes the Applied Biosystems cycle sequencing chemistries, the dyes used in them, and how to choose a sequencing chemistry.

Topic	See page
Dye Terminator Cycle Sequencing Kits	2-2
Dye Primer Cycle Sequencing Kits	2-8
Dye Spectra	2-12
Chemistry/Instrument/Filter Set Compatibilities	2-13
Dye/Base Relationships for Sequencing Chemistries	2-14
Choosing a Sequencing Chemistry	2-15

Dye Terminator Cycle Sequencing Kits

Rhodamine Dye Terminators

The rhodamine dye terminators have the following dye labels. The structures of the rhodamine dye terminators are shown in Figure 2-1.

Terminator	Dye Label
A	R6G
C	ROX
G	R110
T	TAMRA

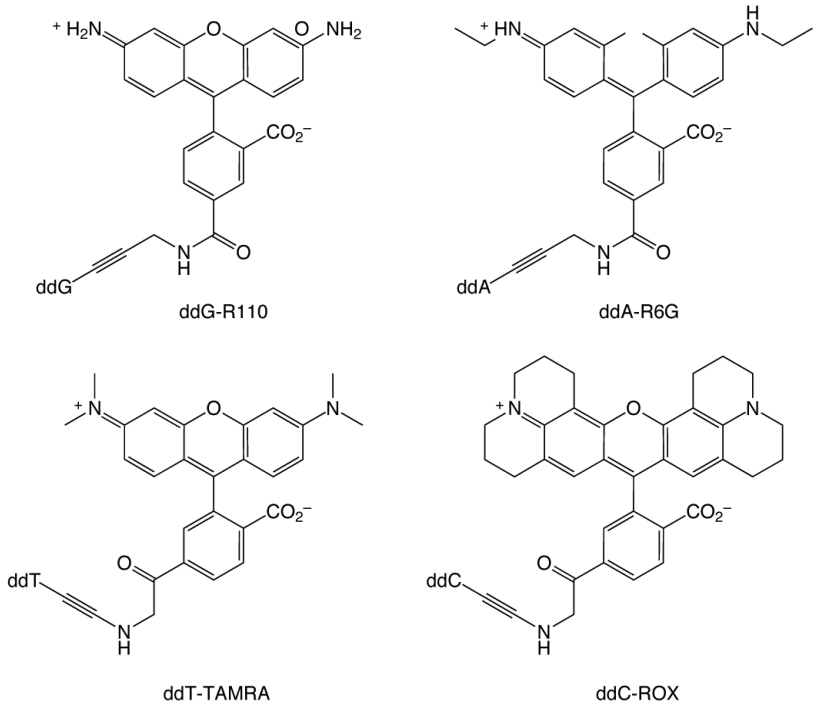

Figure 2-1 Rhodamine dye terminators

Rhodamine Dye Terminator Kits

The ABI PRISM™ Dye Terminator Cycle Sequencing Kits combine AmpliTaq® DNA Polymerase, FS, rhodamine dye terminators, and all the required components for the sequencing reaction.

Note Throughout this manual, these kits will be referred to as “rhodamine dye terminators.”

The concentrations of the dye-labeled dideoxynucleotides and deoxynucleotides in the dNTP mix have been optimized to give a balanced distribution of signal above 700 bases. The dNTP mix includes dITP in place of dGTP to minimize band compressions.

In the Ready Reaction format, the dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, r*Tth* pyrophosphatase, magnesium chloride, and buffer are premixed into a single tube of Ready Reaction Mix and are ready to use. These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, or on polymerase chain reaction (PCR) fragments.

In the Core Kit format, the reagents are supplied in individual tubes to maximize kit flexibility. For convenience when sequencing large quantities of templates, the reagents can be premixed and stored.

The cycle sequencing protocols are optimized for GeneAmp® PCR Instrument Systems thermal cyclers, the CATALYST™ 800 Molecular Biology LabStation, and the ABI PRISM® 877 Integrated Thermal Cycler. For more information, refer to the *ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit Protocol* (P/N 402078) or the *ABI PRISM Dye Terminator Cycle Sequencing Core Kit Protocol* (P/N 402116).

dRhodamine Terminators

Applied Biosystems has designed new dichlororhodamine (dRhodamine) dye terminators to give more even peak heights than the rhodamine dye terminators (Rosenblum *et al.*, 1997). The new dyes have narrower emission spectra, giving less spectral overlap and therefore less noise (Figure 2-7 on page 2-12).

The new dRhodamine dye terminators have the following dye labels. The dye terminator structures are shown in Figure 2-2.

Terminator	Dye Label
A	dichloro[R6G]
C	dichloro[TAMRA]
G	dichloro[R110]
T	dichloro[ROX]

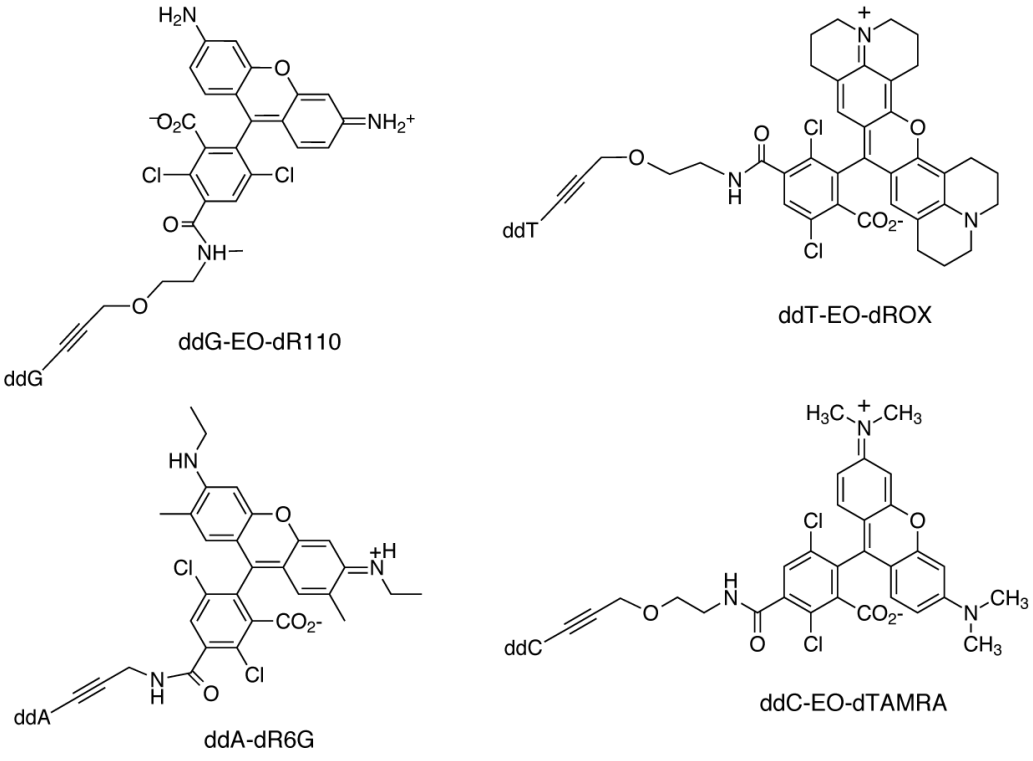

Figure 2-2 dRhodamine terminators

Three of the four dRhodamine terminators use the new ethylene oxide (EO) linker to attach the dye to the dideoxynucleotide. This improves the incorporation of the dye-labeled terminators by the AmpliTaq DNA Polymerase, FS enzyme.

Data collected in Applied Biosystems laboratories shows more uniform signal intensities with the new dyes and a reduction of the weak G after A pattern that is a problem with the rhodamine dye terminators.

With less noise, better signal uniformity, and more even peak heights, the new dRhodamine dye terminators can give better sequencing results than the rhodamine dye terminators (Figure 2-3).

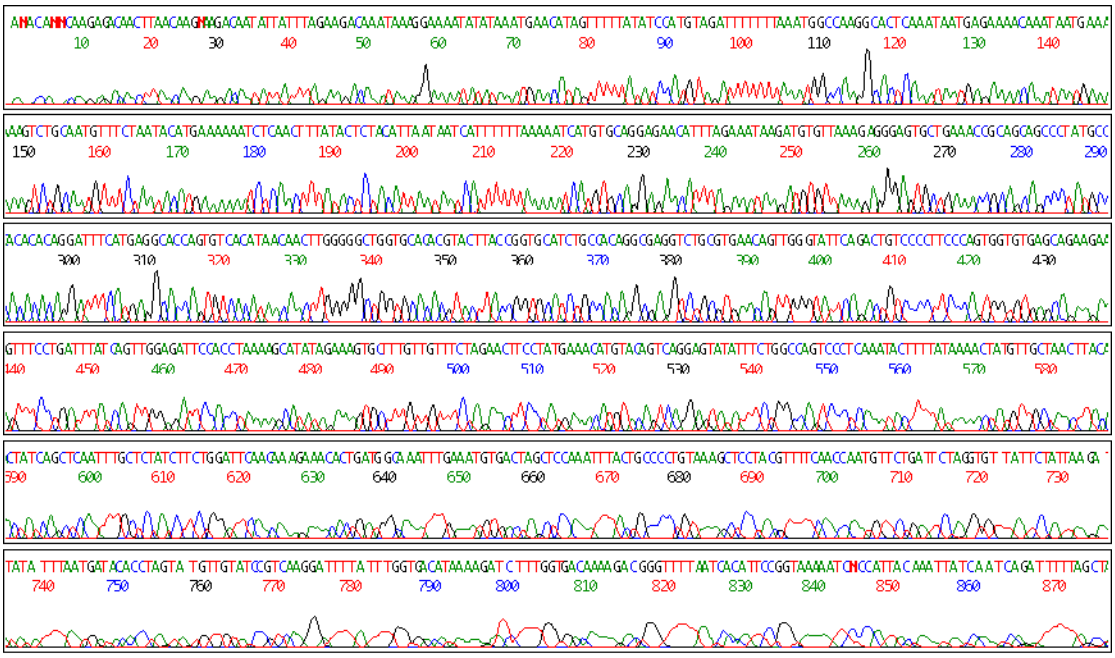

Figure 2-3 Sequence data obtained from a plasmid with dRhodamine terminators. Reactions were run on an ABI PRISM® 377 DNA Sequencer with a 48-cm well-to-read gel.

dRhodamine Terminator Ready Reaction Kits

The ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kits combine AmpliTaq DNA Polymerase, FS and the new dRhodamine dye terminators. In the Ready Reaction format, the dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, r*Tth* pyrophosphatase, magnesium chloride, and buffer are premixed into a single tube of Ready Reaction Mix and are ready to use. The dNTP mix includes dITP in place of dGTP to minimize band compressions.

These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, or on polymerase chain reaction (PCR) fragments. The cycle sequencing protocols are optimized for GeneAmp PCR Instrument Systems thermal cyclers, the CATALYST 800 Molecular Biology LabStation, and the ABI PRISM 877 Integrated Thermal Cycler.

For more information, refer to the *ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit Protocol* (P/N 403041).

Instrument Platforms

The ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kits are for use with the ABI PRISM 310 Genetic Analyzer and ABI PRISM 377 DNA Sequencer (all models).[1]

These kits can also be used with ABI™ 373 DNA Sequencers[2] on which the new ABI PRISM BigDye Filter Wheel has been installed. Refer to the *ABI PRISM BigDye Filter Wheel User Bulletin* (P/N 4304367) for more information.

IMPORTANT This kit is not designed for use with ABI 373 DNA Sequencers and ABI 373 DNA Sequencers with XL Upgrade that do not have the ABI PRISM BigDye Filter Wheel.

BigDye Terminators

Applied Biosystems has developed a set of dye terminators labeled with novel, high-sensitivity dyes (Rosenblum *et al.*, 1997). The new dye structures contain a fluorescein donor dye, *e.g.*, 6-carboxyfluorescein (6-FAM), linked to one of four dichlororhodamine (dRhodamine) acceptor dyes. The excitation maximum of each dye label is that of the fluorescein donor, and the emission spectrum is that of the dRhodamine acceptor (Figure 2-7 on page 2-12).

The donor dye is optimized to absorb the excitation energy of the argon ion laser in the Applied Biosystems DNA sequencing instruments. The linker affords extremely efficient energy transfer (quantum efficiency nearly 1.0, *i.e.*, 100%) between the donor and acceptor dyes. The BigDye™ terminators are 2–3 times brighter than the rhodamine dye terminators when incorporated into cycle sequencing products.

The BigDye terminators are labeled with the following dRhodamine acceptor dyes:

Terminator	Acceptor Dye
A	dichloro[R6G]
C	dichloro[ROX]
G	dichloro[R110]
T	dichloro[TAMRA]

Note The individual dRhodamine dye structures are shown in Figure 2-2 on page 2-3.

The BigDye terminators also have narrower emission spectra than the rhodamine dye terminators, giving less spectral overlap and therefore less noise (Figure 2-7 on page 2-12). The brighter signal and decreased noise provide an overall 4–5X gain in signal-to-noise ratio (Figure 2-4 on page 2-6).

- The nucleotide/dideoxynucleotide mixes have been optimized to give longer, more accurate reads above 700 bases.
- Large templates can be sequenced more readily. One such application is BAC end sequencing.
- Reactions using half the amount of Ready Reaction Premix can be run on some templates, such as PCR products and plasmids (see page 3-22).

1. Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.
2. Includes the ABI 373 and ABI 373 with XL Upgrade instruments.

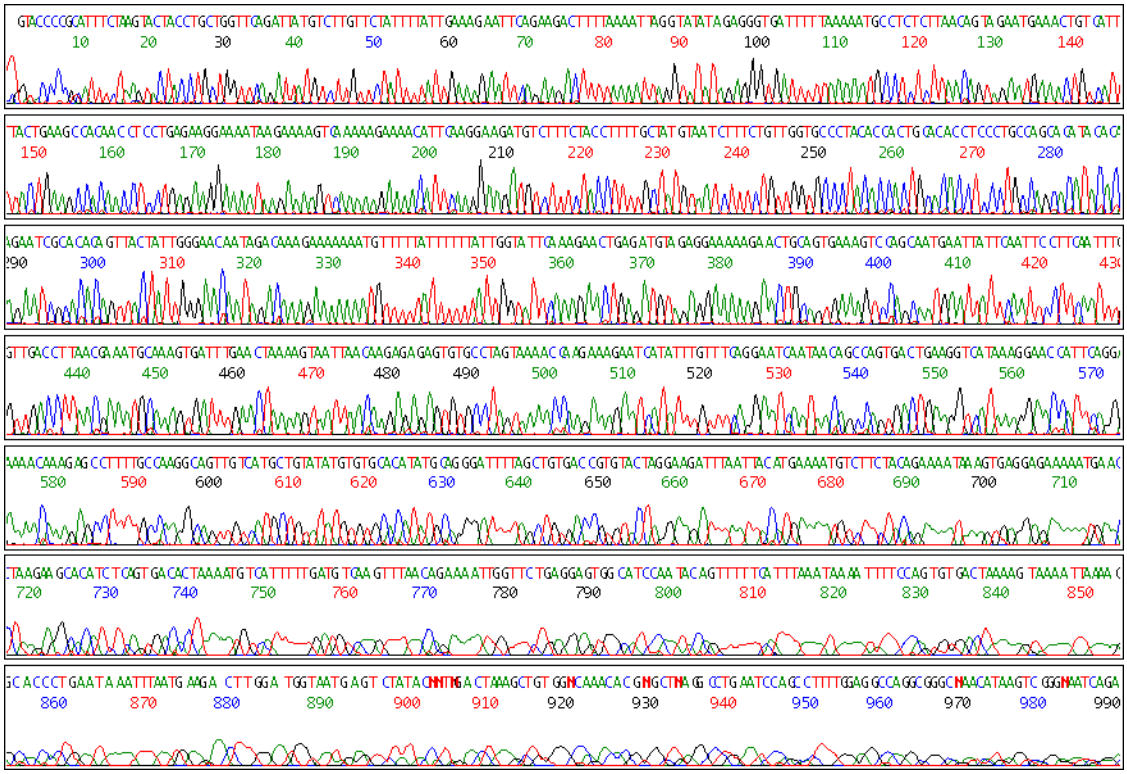

Figure 2-4 Sequence data obtained from a plasmid with BigDye terminators. Reactions were run on an ABI PRISM 377 DNA Sequencer with a 5.25% PAGE-PLUS, 48-cm well-to-read gel.

BigDye Terminator Ready Reaction Kits

The ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits combine AmpliTaq DNA Polymerase, FS, the new BigDye terminators, and all the required components for the sequencing reaction.

In the Ready Reaction format, the dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, r*Tth* pyrophosphatase, magnesium chloride, and buffer are premixed into a single tube of Ready Reaction Mix and are ready to use. These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, on polymerase chain reaction (PCR) fragments, and on large templates, *e.g.*, BAC clones.

The dNTP mix includes dITP in place of dGTP to minimize band compressions.The dNTP mix also uses dUTP in place of dTTP. dUTP improves the incorporation of the T terminator and results in a better T pattern.

The cycle sequencing protocols are optimized for GeneAmp PCR Instrument Systems thermal cyclers, the CATALYST 800 Molecular Biology LabStation, and the ABI PRISM 877 Integrated Thermal Cycler.

For more information, refer to the *ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit Protocol* (P/N 4303237).

Instrument Platforms

The ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits are for use with the ABI PRISM 310 Genetic Analyzer and ABI PRISM 377 DNA Sequencer (all models).[1]

These kits can also be used with ABI 373 DNA Sequencers[2] on which the new ABI PRISM BigDye Filter Wheel has been installed. Refer to the *ABI PRISM BigDye Filter Wheel User Bulletin* (P/N 4304367) for more information.

IMPORTANT This kit is not designed for use with ABI 373 DNA Sequencers and ABI 373 DNA Sequencers with XL Upgrade that do not have the ABI PRISM BigDye Filter Wheel.

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1. Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.
2. Includes the ABI 373 and ABI 373 with XL Upgrade instruments.

Dye Primer Cycle Sequencing Kits

Fluorescein/ Rhodamine Dye Primers

The fluorescein/rhodamine dye primers (see note below) have the following dye labels:

Primer	Dye Label
A	JOE
C	5-FAM
G	TAMRA
T	ROX

The structures of the fluorescein/rhodamine dye primers are shown in Figure 2-5. Note that 5-FAM and JOE are fluorescein dyes and TAMRA and ROX are rhodamine dyes. The emission spectra of the dyes are shown in Figure 2-8 on page 2-12.

Note Throughout this manual, this chemistry will be referred to as “fluorescein/rhodamine dye primer” to distinguish it from BigDye primer chemistry.

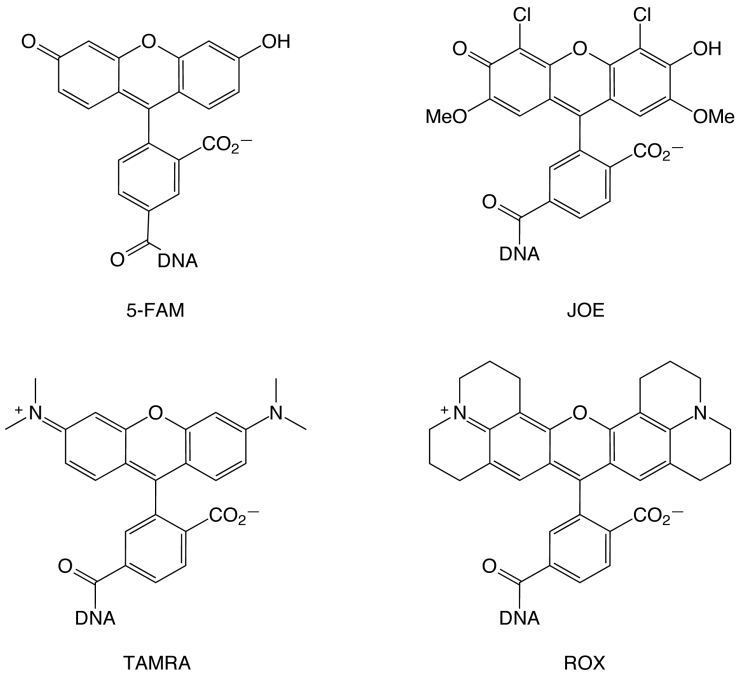

Figure 2-5 Fluorescein/rhodamine dye primers

Fluorescein/ Rhodamine Dye Primer Kits

The ABI PRISM Dye Primer Cycle Sequencing Kits include AmpliTaq DNA Polymerase, FS, dye-labeled primers and all the required components for the sequencing reaction.

The deoxy- and dideoxynucleotide ratios in the dNTP mix have been optimized to give a balanced distribution of signal between base 10 and base 700+ after the primer. The dNTP mix includes 7-deaza-dGTP in place of dGTP to minimize band compressions.

In the Ready Reaction format, the dye-labeled primers, deoxynucleoside triphosphates, dideoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, r*Tth*

pyrophosphatase, magnesium chloride, and buffer are premixed into A, C, G, and T Ready Reaction cocktails to eliminate time-consuming reagent preparation. These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, or on polymerase chain reaction (PCR) fragments.

In the Core Kit format, the reagents are supplied in individual tubes to maximize kit flexibility. For convenience when sequencing large quantities of templates, the reagents can be premixed and stored for later use.

The cycle sequencing protocols are optimized for GeneAmp PCR Instrument Systems thermal cyclers, the CATALYST 800 Molecular Biology LabStation, and the ABI PRISM 877 Integrated Thermal Cycler.

Note We do not recommend using fluorescein/rhodamine dye primers with the POP-6™ polymer on the ABI PRISM 310 Genetic Analyzer.

For more information, refer to the *ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit Protocol* (P/N 402113) or the *ABI PRISM Dye Primer Cycle Sequencing Core Kit Protocol* (P/N 402114).

BigDye Primers

Applied Biosystems has developed a set of dye primers labeled with novel, high-sensitivity dyes (Lee *et al*., 1997). The new dye structures contain a fluorescein donor dye, *e.g*., 6-carboxyfluorescein (6-FAM), linked to one of four dichlororhodamine (dRhodamine) acceptor dyes. The excitation maximum of each dye label is that of the fluorescein donor, and the emission spectrum is that of the dRhodamine acceptor (Figure 2-7 on page 2-12).

The donor dye is optimized to absorb the excitation energy of the argon ion laser in the Applied Biosystems DNA sequencing instruments. The linker affords extremely efficient energy transfer (quantum efficiency nearly 1.0, *i.e*., 100%) between the donor and acceptor dyes. Hence, the BigDye™ primers are 2–3 times brighter than the fluorescein/rhodamine dye primers when incorporated into cycle sequencing products.

The BigDye primers are labeled with the following dRhodamine acceptor dyes:

Primer	Acceptor Dye
A	dichloro[R6G]
C	dichloro[R110]
G	dichloro[TAMRA]
T	dichloro[ROX]

Note The individual dRhodamine dye structures are shown in Figure 2-2 on page 2-3. The BigDye primers use the same dyes as the BigDye terminators.

The BigDye primers also have narrower emission spectra than the fluorescein/rhodamine dye primers, giving less spectral overlap and therefore less noise (Figure 2-7 on page 2-12). The brighter signal and decreased noise provide an overall 4–5X gain in signal-to-noise ratio, giving added flexibility in sequencing applications:

- The A, C, G, and T reactions are carried out in a 1:1:1:1 ratio.
- The nucleotide/dideoxynucleotide mixes have been optimized to give longer, more accurate reads above 700 bases.

- Large templates can be sequenced more readily. One such application is BAC end sequencing.
- Reactions using half the amount of Ready Reaction Premix can be run on some templates, such as PCR products and plasmids (see page 3-25).
- In some cases, reactions can be loaded onto the sequencing instrument without precipitation (see page 3-49).

Figure 2-6 shows BigDye primer sequencing data.

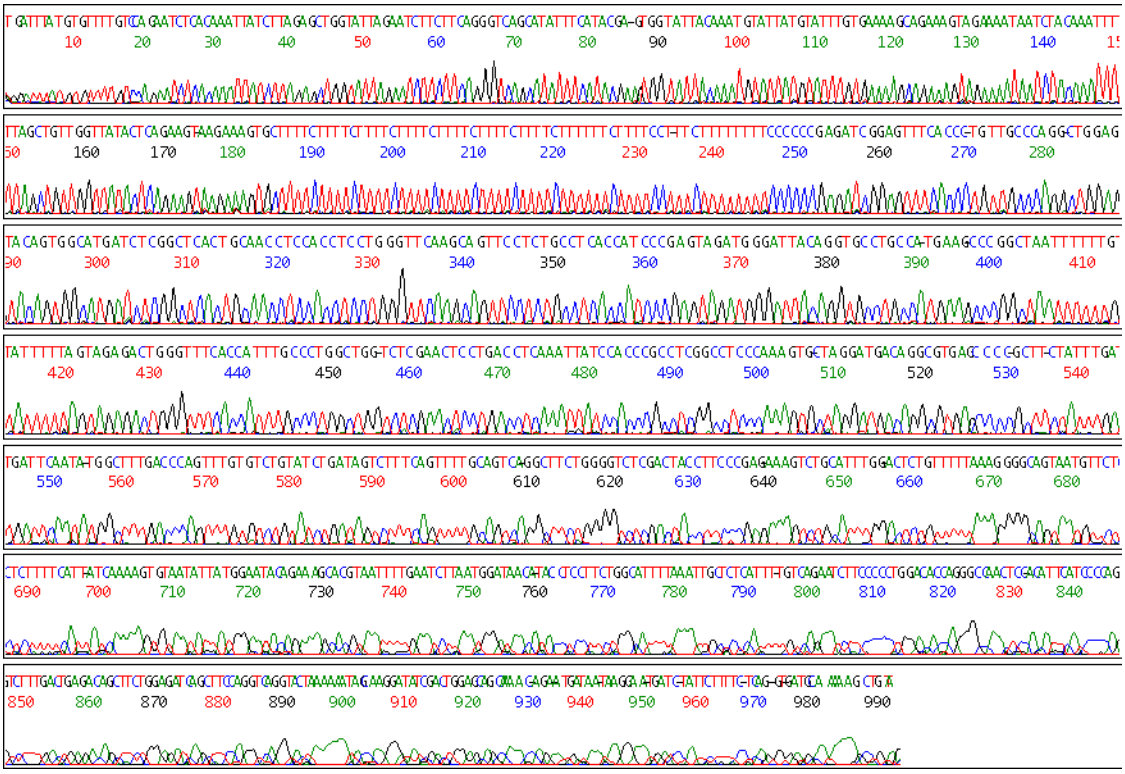

Figure 2-6 Sequence data obtained from a plasmid with BigDye primers. Reactions were run on an ABI PRISM 377 DNA Sequencer with a 5.25% PAGE-PLUS, 48-cm well-to-read gel.

BigDye Primer Ready Reaction Kits

The ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction Kits combine AmpliTaq DNA Polymerase, FS, the new BigDye primers, and all the required components for the sequencing reaction.

The kits contain nucleotide mixes that have been specifically optimized for AmpliTaq DNA Polymerase, FS. The deoxy- and dideoxynucleotide ratios in the nucleotide mixes have been formulated to give a good signal balance above 700 bases. These formulations also contain 7-deaza-dGTP in place of dGTP to minimize band compressions.

In the Ready Reaction format, the dye-labeled primers, deoxynucleoside triphosphates, dideoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, r*Tth* pyrophosphatase, magnesium chloride, and buffer are premixed into A, C, G, and T Ready Reaction cocktails to eliminate time-consuming reagent preparation. These reagents are suitable for performing fluorescence-based cycle sequencing reactions

on single-stranded or double-stranded DNA templates, on polymerase chain reaction (PCR) fragments, and on large templates, *e.g.*, the ends of BAC clones.

The cycle sequencing protocols are optimized for GeneAmp PCR Instrument Systems thermal cyclers, the CATALYST 800 Molecular Biology LabStation, and the ABI PRISM 877 Integrated Thermal Cycler.

For more information, refer to the *ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction Kit Protocol* (P/N 403057).

Instrument Platforms

The ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction Kits are for use with the ABI PRISM 310 Genetic Analyzer and ABI PRISM 377 DNA Sequencer (all models).[1]

These kits can also be used with ABI 373 DNA Sequencers[2] on which the new ABI PRISM BigDye Filter Wheel has been installed. Refer to the *ABI PRISM BigDye Filter Wheel User Bulletin* (P/N 4304367) for more information.

IMPORTANT This kit is not designed for use with ABI 373 DNA Sequencers and ABI 373 DNA Sequencers with XL Upgrade that do not have the ABI PRISM BigDye Filter Wheel.

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1. Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.
2. Includes the ABI 373 and ABI 373 with XL Upgrade instruments.

Dye Spectra

Rhodamine and dRhodamine Dyes

The normalized emission spectra of the rhodamine and dRhodamine dyes are shown in Figure 2-7. The dRhodamine dyes are used in the ABI PRISM dRhodamine Terminator, BigDye Primer, and BigDye Terminator Cycle Sequencing Ready Reaction Kits.

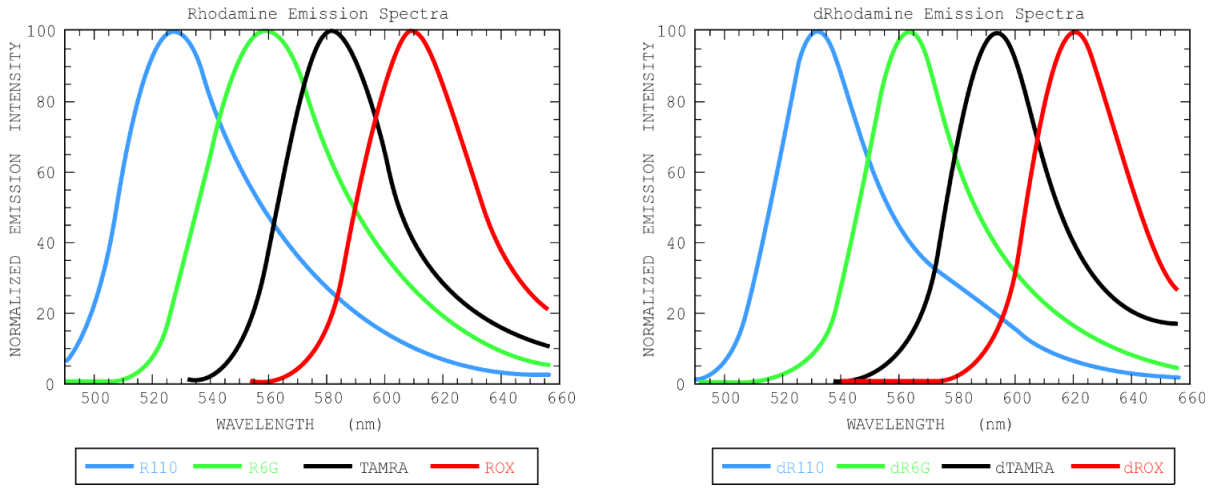

Figure 2-7 Emission spectra of rhodamine and dRhodamine dyes. Note the narrower emission spectra of the dRhodamine dyes.

Fluorescein/ Rhodamine Dyes

The normalized emission spectra of the fluorescein and rhodamine dyes used in the ABI PRISM Dye Primer Cycle Sequencing Kits are shown in Figure 2-8.

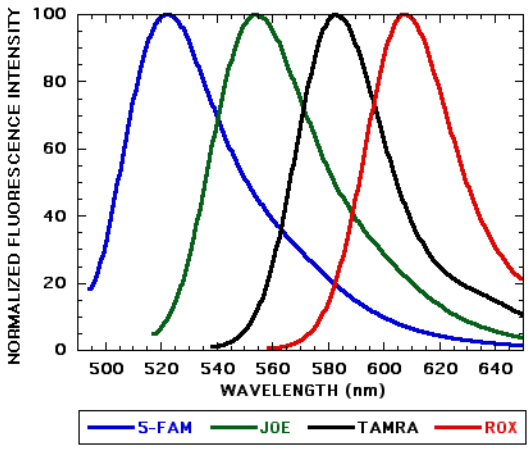

Figure 2-8 Emission spectra of the dyes used in ABI PRISM Dye Primer Cycle Sequencing Kits

Chemistry/Instrument/Filter Set Compatibilities

Chemistry and Instrument Compatibilities

Table 2-1 shows which chemistries can be used on which instruments.

Table 2-1 Chemistry/Instrument Compatibilities

Instrument	Sequencing Chemistry				
	Rhodamine Dye Terminator	**dRhodamine Terminator**	**BigDye Terminator**	**Fluorescein/ Rhodamine Dye Primer**	**BigDye Primer**
ABI 373[a]	yes	no	no	yes	no
ABI 373[a] with BigDye Filter Wheel	no	yes	yes	no	yes
ABI PRISM® 310 and ABI PRISM 377[b]	yes	yes	yes	yes	yes

a. Includes the ABI 373 and ABI 373 with XL Upgrade instruments.

b. All models.

Filter Sets

Table 2-2 shows the filter sets and virtual filter sets that are used with the Applied Biosystems cycle sequencing chemistries.

Table 2-2 Sequencing Chemistries and Filter Sets

Chemistry	Filter Set		Virtual Filter Set
	ABI 373[a]	**ABI 373[a] with BigDye Filter Wheel**	**ABI PRISM 310 and ABI PRISM 377[b]**
Rhodamine Dye Terminator	A	Cannot use these chemistries with this instrument configuration	A
Fluorescein/ Rhodamine Dye Primer			
dRhodamine Terminator	Cannot use these chemistries with this instrument configuration	A	E
BigDye™ Terminator			
BigDye™ Primer			

a. Includes the ABI 373 and ABI 373 with XL Upgrade instruments.

b. All models.

Dye/Base Relationships for Sequencing Chemistries

Overview During the development of a new sequencing chemistry, alternative dye/base relationships are investigated to see which produces the most uniform signal in the analyzed data. For this reason, different sequencing chemistries may have different dye/base relationships.

The Sequencing Analysis software compensates for this when the correct dye set/primer (mobility) file is used (see page 6-3). The software always displays A as green, C as blue, G as black, and T as red in the electropherogram view of analyzed data.

Dye/Base Relationships

Table 2-3 dRhodamine Terminator Dye/Base Relationships

Terminator	Dye	Color of Unanalyzed Data on Electropherogram[a]	Color of Raw Data on Gel Image[b]	Color of Analyzed Data on Electropherograms[c]
A	dR6G	green	green	green
C	dTAMRA	black	yellow	blue
G	dR110	blue	blue	black
T	dROX	red	red	red

Table 2-4 Rhodamine Dye Terminator and BigDye Terminator Dye/Base Relationships

Terminator	Dye		Color of Unanalyzed Data on Electropherogram[a]	Color of Raw Data on Gel Image[b]	Color of Analyzed Data on Electropherograms[c]
	Rhodamine Dye Terminator	BigDye Terminator			
A	R6G	dR6G	green	green	green
C	ROX	dROX	red	red	blue
G	R110	dR110	blue	blue	black
T	TAMRA	dTAMRA	black	yellow	red

Table 2-5 Fluorescein/Rhodamine Dye Primer and BigDye Primer Dye/Base Relationships

Base	Dye		Color of Unanalyzed Data on Electropherogram[a]	Color of Raw Data on Gel Image[b]	Color of Analyzed Data on Electropherograms[c]
	Fluorescein/ Rhodamine Dye Primer	BigDye Primer			
A	JOE	dR6G	green	green	green
C	5-FAM	dR110	blue	blue	blue
G	TAMRA	dTAMRA	black	yellow	black
T	ROX	dROX	red	red	red

a. ABI PRISM 310 Genetic Analyzer.

b. ABI 373 and ABI PRISM 377 DNA Sequencers. See Table 2-1 on page 2-13 for chemistry/instrument compatibilities.

c. All instruments. See Table 2-1 on page 2-13 for chemistry/instrument compatibilities.

Choosing a Sequencing Chemistry

Overview

Although all of the sequencing chemistries are relatively versatile, some are better choices than others for specific types of templates. No single chemistry works with every template. While you can choose a single kit for most work, a second chemistry or modifications to the standard protocol of the main sequencing chemistry may be necessary. See “Troubleshooting DNA Sequence Composition Problems” on page 7-30 for more information.

ABI PRISM 310, ABI 373 with BigDye Filter Wheel, and ABI PRISM 377

We generally recommend the BigDye terminators because of their optimal signal-to-noise characteristics, ease of use, and versatility. Table 2-6 shows the chemistry recommendations for various applications.

Table 2-6 ABI PRISM 310, ABI 373 with BigDye Filter Wheel, and ABI PRISM 377 Chemistry Recommendations

	dRhodamine Terminator	BigDye Terminator	BigDye Primer
DNA Sequencing Application			
De novo sequencing—high throughput	S	R[a]	R
De novo sequencing—mid-to-low throughput	S	R	S
Comparative sequencing (germline mutations 50:50 heterozygotes)	S	R	R
Comparative sequencing (somatic mutations 30:70 heterozygotes)	N	S	R
Comparative sequencing (somatic mutations 10:90 heterozygotes)	N	N	S
Gene walking (custom primers)	S	R	N
Shotgun sequencing (universal primers, M13)	S	R	R
Deletion clone sequencing (universal primers)	S	R	R
Gap closure (custom primers)	S	R	N
DNA Sequence Context			
GC-rich >65%	S	R	S
AT-rich >65%	R	R	R
GT-rich regions	R	N	R
Homopolymer A or T >25 bp[b]	R	N	R
Template			
Plasmid (<15 kb)	R	R	R
M13	R	R	R
BAC, cosmid, lambda, large PCR product	S	R	S
Bacterial genomic DNA	N	R	N
PCR amplicon	R	R	R
PCR amplicon (heterozygous 50:50)	S	R	R
PCR amplicon (heterozygous 30:70)	N	S	R
PCR amplicon (heterozygous 10:90)	N	N	S

a. R = recommended, S = satisfactory, N = not recommended

b. All cycle sequencing chemistries can have difficulties with homopolymers >40 bp.

The dRhodamine terminators are useful for templates with long homopolymer (>25 bases) stretches or templates with GT-rich motifs. However, the dRhodamine terminators produce weaker signals than the BigDye chemistries. More of the sample must be loaded to ensure adequate signal is available. This is especially important for running 48-, 64- and 96-lane gels on the ABI PRISM 377 DNA Sequencer, where less signal is detected because the lanes are narrower. To compensate for the decreased signal strength with dRhodamine terminators, increase the CCD gain from 2 to 4.

ABI 373 DNA Sequencer

Table 2-7 provides a list of sequencing applications and suggests kits that best suit them.

Table 2-7 ABI 373 Chemistry Recommendations

	Rhodamine Dye Terminator	Fluorescein/ Rhodamine Dye Primer
DNA Sequencing Application		
De novo sequencing—high throughput	R[a]	R
De novo sequencing—mid-to-low throughput	R	S
Comparative sequencing (germline mutations 50:50 heterozygotes)	N	R
Comparative sequencing (somatic mutations 30:70 heterozygotes)	N	R
Comparative sequencing (somatic mutations 10:90 heterozygotes)	N	S
Gene walking (custom primers)	R	N
Shotgun sequencing (universal primers, M13)	R	R
Deletion clone sequencing (universal primers)	R	R
Gap closure (custom primers)	R	N
DNA Sequence Context		
GC-rich >65%	R	S
AT-rich >65%	R	R
GT-rich regions	S	R
Homopolymer A or T >25 bp[b]	S	R
Template		
Plasmid (<15 kb)	R	R
M13	R	R
BAC, cosmid, lambda, large PCR product	S	S
Bacterial genomic DNA	N	N
PCR amplicon	R	R
PCR amplicon (heterozygous 50:50)	N	R
PCR amplicon (heterozygous 30:70)	N	R
PCR amplicon (heterozygous 10:90)	N	R

a. R = recommended, S = satisfactory, N = not recommended

b. All cycle sequencing chemistries can have difficulties with homopolymers >40 bp.

Performing DNA Sequencing Reactions 3

Overview

Factors That Affect Data Quality

With careful template preparation and sequencing techniques, you can obtain reliable sequence data for both dye primer and dye terminator chemistries. This section describes the factors affecting data quality, how they can be controlled during sample preparation and the sequencing reactions, and how some fundamental errors can be avoided and corrected:

Topic	See page
DNA Template Preparation	3-2
Sequencing PCR Templates	3-10
DNA Template Quality	3-15
DNA Template Quantity	3-17
Primer Design and Quantitation	3-18
Reagent and Equipment Considerations	3-20
Preparing Cycle Sequencing Reactions	3-21
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Preparing and Loading Samples for Gel Electrophoresis	3-50
Preparing and Loading Samples for Capillary Electrophoresis	3-53

DNA Template Preparation

Overview

The DNA purification method used can affect the quality of the template. Some recommendations for purifying DNA templates are given below.

Prepare adequate template to check purity (see "Determining DNA Quality" on page 3-16), to quantitate the DNA accurately (see "Quantitating DNA" on page 3-17), and to perform the sequencing reactions. The recommended quantities for sequencing reactions are shown in Table 3-1 on page 3-17.

Single-stranded DNA Templates

You can use the following methods to prepare single-stranded templates such as M13:

- QIAGEN (http://www.qiagen.com) QIAprep Spin M13 Kit (P/N 27704, 50 reactions)
- High-throughput (ThermoMAX procedure, see below)
- PEG precipitation followed by phenol extraction (see below)

ThermoMAX Procedure

Cells infected with recombinant M13 phage are grown in liquid medium. The growth medium is clarified by centrifugation and PEG precipitation. The phage particles are resuspended in buffer and then heated to release the single-stranded DNA.

Reagents and equipment required:

- 2X TY medium, pH 7.2–7.4

Step	Action
1	Combine the following: ♦ Bactotryptone, 16.0 g ♦ Yeast extract, 5.0 g ♦ NaCl, 5.0 g Make up to 1 L in autoclaved water.
2	Adjust the pH to 7.2–7.4 with NaOH.

- PEG solution (20% PEG, 2.5 M NaCl)

  Make up fresh as needed from equal volumes of 40% PEG (in deionized water) and 5 M NaCl stocks.

- TTE buffer (0.25% v/v Triton X-100, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

Step	Action
1	Combine the following: ♦ Tris-HCl (pH 8.0), 1 M, 500 µL ♦ Na_2EDTA, 0.5 M, 10 µL ♦ Triton X-100, 250 µL
2	Make up to 50 mL in deionized water.

- 96-cap sealer (Beckman)
- Adhesive-backed aluminum foil tape (3M Scotch Tape 425-3)[1]

- Centrifuge with 96-tube tray adapter
- Sterile 1.2-mL culture tubes, 96/box
- Sterile toothpicks

To grow M13-infected cells:

Step	Action
1	Inoculate 250 mL of 2X TY with 1 mL of JM101.
2	Transfer 0.8 mL of the JM101 culture to each of 96 1.2-mL mini-tubes (one box).
3	Wearing clean gloves, pick M13 plaques using sterile toothpicks. Drop each toothpick into a culture tube. Remove toothpicks after all 96 have been picked.
4	Cover the rack of tubes with the provided cover. Shake the tubes at 37 °C for 18–19 hours.

To precipitate M13 phage particles with PEG:

Step	Action
1	Spin the cultures in a centrifuge at 3250 rpm for 15 minutes to pellet the cells.
2	While the cells are pelleting, add 120 µL of PEG solution to each tube in a second 96-tube box.
3	Carefully transfer 0.6 mL of M13 supernatant from each tube from the first box to the corresponding tube in the second box.
4	Cover the tubes with a Beckman 96-cap sealer, and invert several times to mix.
5	Leave the tubes at room temperature for 30 minutes, then chill at 2–6 °C for 30 minutes.
6	Spin the cultures in a centrifuge at 3250 rpm for 15 minutes to pellet the M13 particles.
7	When centrifugation is finished, check for pellets by discarding the supernatant from a single row. Be sure to place the row back in place correctly. If the pellets are present then discard the supernatant from the rest of the samples.
8	Place the inverted tubes on a paper towel for a few minutes to drain.
9	With the tubes still inverted, place them on a dry paper towel in the centrifuge carrier. Spin the inverted tubes in a centrifuge at 300 rpm for 3–5 minutes to remove all traces of PEG. After spinning, check to see that the pellets have stayed at the bottoms of the tubes.

To extract the M13 DNA:

Step	Action
1	Add 20 µL of TTE buffer to each tube.
2	Seal the tubes with aluminum foil tape and vortex vigorously. **Note** Ensure that the pellet is well suspended.
3	Heat the tubes at 80 °C for 10 minutes in a water bath.

1. Contact 3M in the USA at (800) 364-3577 for your local 3M representative. Use of other tapes may result in leakage or contamination of the sample.

To extract the M13 DNA: *(continued)*

Step	Action
4	While the samples are heating, add 70 µL of deionized water to each well of a fresh 96-well plate.
5	When heating is complete, spin the samples briefly in a centrifuge to return the liquid to the bottoms of the tubes. Transfer the samples to the 96-well plate containing the deionized water.
6	Cover the samples with foil tape and place in a non-frost-free, –15 to –25 °C freezer.

For more information, refer to the Washington University School of Medicine Genome Sequencing Center *Genome Sequencing Manual* (http://genome.wustl.edu/gsc/manual/protocols/M13_ThermoMAX_prep.html).

Preparing DNA from M13 Phage by PEG Precipitation and Phenol Extraction

Cells infected with recombinant M13 phage are grown in liquid medium. After clarification of the growth medium by centrifugation and PEG precipitation, single-stranded DNA is extracted from the phage particles in the supernatant.

Reagents and equipment required:

- 2X TY medium, pH 7.2–7.4

Step	Action
1	Combine the following: ♦ Bactotryptone, 16.0 g ♦ Yeast extract, 5.0 g ♦ NaCl, 5.0 g Make up to 1 L in autoclaved water.
2	Adjust the pH to 7.2–7.4 with NaOH.

- Chloroform

! WARNING ! CHEMICAL HAZARD. Chloroform is extremely toxic and a potential human carcinogen. This chemical is highly corrosive to skin and eyes. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.

- Ethanol, 95%
- Ethanol, 70%
- PEG solution (20% PEG, 2.5 M NaCl)

  Make up fresh as needed from equal volumes of 40% PEG (in deionized water) and 5 M NaCl stocks.
- Sodium acetate (NaOAc), 3 M, pH 5.2
- TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
- $TE_{0.1}$ buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0)
- Tris buffer (10 mM, pH 8.0)
- Tris-saturated phenol, pH >7.6

! WARNING ! CHEMICAL HAZARD. Phenol is a highly toxic, combustible, and vesicant chemical that causes burns and is readily absorbed through the skin. It is extremely destructive to mucous membranes, eyes, and skin. Inhalation and ingestion can cause CNS, liver, pancreas, and spleen damage, and can be fatal. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.

- 1.5-mL microcentrifuge tubes
- Sterile 10-mL tubes (centrifugable at 10,000 × *g*)
- Sterile cotton-tipped swabs
- Sterile Pasteur pipettes

To grow M13-infected cells:

Step	Action
1	Inoculate 10 mL of 2X TY medium with a single colony of *E. coli* grown on glucose minimal agar.
2	Shake overnight at 37 °C.
3	Dilute an aliquot of the culture 1:100 in 2X TY medium and dispense 5-mL aliquots into sterile 10-mL tubes.
4	Using the tip of a Pasteur pipette, add one agar plug from the recombinant plaque to each 10-mL tube.
5	Shake the 10-mL tubes at 37 °C for 6–7 hours. **Note** Incubation longer than 6–7 hours may complicate purification.

To clarify the culture by centrifugation and PEG precipitation:

Step	Action
1	Spin the culture in a centrifuge at 2500 × *g* for 10 minutes. Transfer the supernatant containing the M13 particles to a sterile tube. **Note** The supernatant can be stored for up to 1 month at 2–6 °C.
2	Spin the supernatant in a centrifuge for 10 minutes at 5000 × *g*. Transfer the resulting supernatant to a fresh 10-mL tube that will withstand centrifugation at 10,000 × *g*. **Note** This second centrifugation is necessary even if supernatants have not been stored. **Note** The supernatant from the second centrifugation can be stored after addition of 50 µL chloroform/mL of supernatant. The chloroform kills any remaining bacteria and destroys any enzymes without damaging the DNA.
3	Add 1 mL PEG solution to aggregate the M13 particles. Mix the culture and PEG solution well and allow the mixture to stand at 2–6 °C for 30 minutes.
4	Centrifuge the PEG/phage suspension for 10 minutes at 10,000 × *g* to sediment the M13 particles.
5	Aspirate and discard the supernatant. Invert the tube to drain excess liquid, and carefully remove any remaining traces of supernatant with cotton-tipped swabs. **IMPORTANT** Removal of all supernatant is critical. PEG and salt inhibit sequencing reactions. The PEG/phage pellet should be visible at this stage of preparation.

To extract DNA from the phage particles:

Step	Action
1	Resuspend the pellet in 400 µL TE buffer. Transfer the suspension to microcentrifuge tubes.
2	Extract the suspension twice with Tris-saturated phenol: a. Add 400 µL of Tris-saturated phenol. b. Vortex to mix. c. Centrifuge the extraction mix for 1 minute in a microcentrifuge to separate the organic and aqueous phases. d. Remove the upper aqueous phase, and transfer it to a fresh microcentrifuge tube. **IMPORTANT** Take care not to disturb the aqueous/organic interface, which contains lipid and denatured protein that can inhibit the DNA polymerase used for cycle sequencing reactions.
3	Extract the aqueous phase twice with chloroform to remove phenol, which can affect sequencing data: a. Add 400 µL of chloroform. b. Vortex each extraction until all cloudiness disappears. c. Spin the emulsion 30 seconds in a microcentrifuge to separate the phases. d. Transfer the upper aqueous phase to a fresh microcentrifuge tube. Remove any remaining chloroform by vacuum centrifugation.
4	Precipitate the DNA: a. Add 40 µL of 3 M sodium acetate and 1 mL of 95% ethanol. b. Invert the tube to mix. c. Chill the mixture for at least 20 minutes at –70 °C or overnight at –15 to –25 °C.
5	Spin the mixture in a microcentrifuge for 15 minutes at maximum speed. Aspirate all of the supernatant and discard.
6	Wash the pellet with 70% ethanol: a. Add 250 µL of 70% ethanol to the pellet. b. Spin in a microcentrifuge at maximum speed for 5 minutes. c. Carefully draw off the ethanol and dry the pellet in a vacuum centrifuge for no more than 5 minutes.
7	Dissolve the DNA pellet in 20 µL of deionized water or $TE_{0.1}$ buffer. Assess the quality of the DNA spectrophotometrically or by agarose gel electrophoresis (see “Determining DNA Quality” on page 3-16). Quantitate the DNA spectrophotometrically (see “Quantitating DNA” on page 3-17).
8	Store the DNA at –15 to –25 °C until needed for sequencing reactions.

Plasmid DNA Templates

When purifying recombinant plasmids in bacteria, plate out the transformants to obtain isolated colonies. Select a single colony and restreak out on a plate. Select an isolated colony from that plate to obtain plasmids with the desired insert.

The optimal procedure for preparing a particular plasmid depends on the particular bacterial strain and the yield of each construct. Good sequencing data has been obtained from the following methods:

- ABI PRISM™ Plasmid Miniprep Kit (P/N 402790 or 402791)

- Cesium chloride (CsCl) banding
- Modified alkaline lysis/PEG precipitation method (see below)
- PureGene DNA Isolation Kit (Gentra Systems, Inc., P/N D-5500A)
- QIAGEN Plasmid Kits (http://www.qiagen.com):
  - Mini (P/N 12123, 25 reactions; 12125, 100 reactions)
  - Midi (P/N 12143, 25 reactions; 12144, 50 reactions; 12145, 100 reactions)
  - Maxi (P/N 12162, 10 reactions; 12163, 25 reactions; 12165, 100 reactions)

Modified Alkaline Lysis/PEG Method

Reagents required:

- Chloroform

! WARNING ! CHEMICAL HAZARD. Chloroform is extremely toxic and a potential human carcinogen. This chemical is highly corrosive to skin and eyes. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.

- Deionized water
- Ethanol, 70%
- GET buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0)
- Isopropanol, 100% (anhydrous)
- PEG 8000, 13% (sterilized by autoclaving, rather than by filtration)

! WARNING ! CHEMICAL HAZARD. Polyethylene glycol 8000 (PEG) can be harmful if inhaled, ingested, or absorbed through the skin. It is irritating to the eyes, skin, and mucous membranes. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.

- Potassium acetate, 3 M, pH 4.8
- RNase A (DNase-free), 10 mg/mL
- Sodium chloride (NaCl), 4 M
- Sodium hydroxide (NaOH), 0.2 N, with 1% SDS (freshly made)

! WARNING ! CHEMICAL HAZARD. Sodium hydroxide (NaOH) can cause severe burns to the skin, eyes, and respiratory tract. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.

! WARNING ! CHEMICAL HAZARD. Sodium dodecyl sulfate (SDS) is a toxic chemical that is harmful to the lungs and internal organs if swallowed. Contact with the eyes can cause serious damage. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.

Note To minimize shearing of contaminating chromosomal DNA, do not use a vortexer during this procedure.

To prepare plasmid DNA by alkaline lysis/PEG treatment:

Step	Action
1	Pellet 1.5-mL aliquots of culture for 1 minute in a microcentrifuge at maximum speed. **Note** A total culture volume of 4.5 mL can be spun down per tube without changing volumes in the procedure. This allows you to achieve a threefold increase in yield while eliminating the need for extra tubes and additional handling.
2	Remove the supernatant by aspiration.
3	Resuspend the bacterial pellet in 200 µL of GET buffer by pipetting up and down.
4	Add 300 µL of freshly prepared 0.2 N NaOH/1% SDS. Mix the contents of the tube by inversion. Incubate on ice for 5 minutes.
5	Neutralize the solution by adding 300 µL of 3.0 M potassium acetate, pH 4.8. Mix by inverting the tube. Incubate on ice for 5 minutes.
6	Remove cellular debris by spinning in a microcentrifuge at maximum speed for 10 minutes at room temperature. Transfer the supernatant to a clean tube.
7	Add RNase A (DNase-free) to a final concentration of 20 µg/mL. Incubate the tube at 37 °C for 20 minutes.
8	Extract the supernatant twice with chloroform: a. Add 400 µL of chloroform. b. Mix the layers by inversion for 30 seconds. c. Centrifuge the tube for 1 minute to separate the phases. d. Transfer the upper aqueous phase to a clean tube.
9	Add an equal volume of 100% isopropanol. Mix the contents of the tube by inversion.
10	Spin the tube in a microcentrifuge at maximum speed for 10 minutes at room temperature.
11	Remove the isopropanol completely by aspiration.
12	Wash the DNA pellet with 500 µL of 70% ethanol. Dry under vacuum for 3 minutes.
13	Dissolve the pellet in 32 µL of deionized water.
14	Add 8.0 µL of 4 M NaCl, then 40 µL of autoclaved 13% PEG 8000.
15	Mix thoroughly, then leave the sample on ice for 20 minutes.
16	Pellet the plasmid DNA by spinning in a microcentrifuge at maximum speed for 15 minutes at 2–6 °C.
17	Carefully remove the supernatant. Rinse the pellet with 500 µL of 70% ethanol.
18	Resuspend the pellet in 20 µL of deionized water. Store at –15 to –25 °C.

BAC DNA Templates

With larger DNA targets such as bacterial artificial chromosomes (BACs), the quality of DNA template is important to the success of the sequencing reaction. Two methods have given good sequencing results:

- Alkaline lysis, with extra phenol extraction and isopropanol precipitation if very clean DNA is desired (Marra *et al.*, 1996)
- Cesium chloride (CsCl) banding

Commercial kits are also available for BAC DNA preparation:

- ProPrep BAC (LigoChem, http://www.ligochem.com)
  - Individual reactions: P/N PLK-100, 100 reactions; PLK-1000, 1000 reactions
  - 96-well plates: P/N PLF-1000, 1 plate; PLF-1000, 10 plates; PLF-2500, 25 plates)
- QIAGEN-tip 100 (QIAGEN: P/N 10043, 25 reactions; 10045, 100 reactions) and QIAGEN-tip 500 (QIAGEN: P/N 10063, 25 reactions; 10065, 100 reactions)

For other BAC DNA preparation protocols, refer to the following Web sites:

- Centre National de Séquençage (CNS, or Génoscope): http://www.cns.fr/externe/arabidopsis/protoBAC.html
- The Institute for Genome Research (TIGR): http://www.tigr.org/softlab/TPFBACmultiprep.052397.html
- University of Oklahoma Advanced Center for Genome Technology (ACGT): http://www.genome.ou.edu/DblAcetateProcV3.html
- Washington University School of Medicine Genome Sequencing Center: http://genome.wustl.edu/gsc/manual/protocols/BAC.html

Sequencing PCR Templates

Overview

This section provides information about preparing and sequencing PCR products, but it is not meant to be a detailed guide to PCR amplification. General information on PCR amplification can be found in the *Guide to PCR Enzymes* (Stock No. 700905) and in the product inserts included with GeneAmp® PCR reagents. For PCR amplification, use GeneAmp® PCR Instrument Systems and GeneAmp PCR Core Reagents.

Although PCR fragments can be difficult to denature with traditional single-temperature sequencing methods, cycle sequencing provides several chances to denature and extend the template, which ensures adequate signal in the sequencing reaction.

Visualize PCR products by agarose gel electrophoresis (see "Determining DNA Quality" on page 3-16).

For more detailed information about PCR sequencing, refer to *Comparative PCR Sequencing, A Guide to Sequencing-Based Mutation Detection* (Stock No. 770901-001). This booklet can also be obtained from the Applied Biosystems WWW site (www.appliedbiosystems.com/techsupport).

PCR Strategies

Single Amplification

In the simplest PCR sequencing case, the target DNA is amplified with a single set of primers and then sequenced using the same primers. For many samples, this works well. For the samples that do not work well with this method, optimization of the PCR amplification may be required (see page 3-12). Optimizing the PCR minimizes the presence of non-specific product bands and ensures adequate yield.

A single PCR amplification is also compatible with the use of a sequencing primer that binds internally (semi-nested or nested) to one or both of the PCR primers. This can be helpful if primer-dimer (primer oligomerization) artifacts are a problem (see Figure 7-31 on page 7-24).

Nested and Semi-Nested PCR

If you encounter difficulty with more complex samples, such as bacterial genomic DNA, use a nested or semi-nested PCR. These techniques are useful when the target is present in small quantity. They offer more specificity, which provides superior sequencing data with reduced background signal.

Both nested and semi-nested PCR require two amplifications. The first amplification is identical for nested and semi-nested, but the second amplification differs as described in the following paragraphs.

- Amplify with one set of PCR primers, which converts a complex sample (such as bacterial genomic DNA) into a non-complex sample consisting of the first PCR product and some side products.
- Nested PCR—Amplify 1% or less of the first PCR reaction product using a second set of PCR primers that hybridize at positions internal to the first set.
- Semi-nested PCR—Only one primer of the second set of PCR primers is internal. The other primer is one of the original PCR primers.

Universal-Tailed PCR Primers

A PCR primer can be synthesized with a universal sequencing primer binding site added to the 5´ end (see Appendix E for universal primer sequences). This allows any PCR product to be sequenced with universal primers.

Universal-tailed PCR primers enable the use of commercially available dye-labeled sequencing primers. This technique is also useful with dye terminator chemistries, because universal sequencing primers have good annealing characteristics. However, the longer PCR primers add to the overall cost of the reactions.

Using universal-tailed primers sometimes results in primer oligomerization. As these products have priming sites present, they can result in noisy data for the first 20–100 bases (see page 7-24). Redesigning the PCR primer, optimizing the PCR amplification further, and employing Hot Start methods can help overcome this situation.

Contaminants That Affect PCR Sequencing

Excess Primers and dNTPs

After PCR amplification, the resulting PCR product is in solution along with PCR primers, dNTPs, enzyme, and buffer components. The method used to prepare the PCR product for sequencing depends on the amounts of these components that are carried over and on the chemistry used for sequencing.

Excess PCR primers carried over from the amplification reaction compete with the sequencing primer for binding sites and reagents in the sequencing reaction. This carryover of PCR primers presents more of a problem in dye terminator chemistries because the dye label is incorporated into the extension product after the primer anneals to the template. If more than one primer is present, multiple dye-labeled sequence ladders are generated, resulting in noisy data.

Excess dNTPs from the amplification reaction can affect the balance of the sequencing reaction, resulting in decreased termination in shorter extension fragments.

Nonspecific PCR Products

Nonspecific PCR products include primer-dimer artifacts and secondary PCR products. The presence of any significant quantity of either in a PCR product can result in poor quality sequencing data. Nonspecific PCR products behave as templates in the sequencing reaction and produce extension products, which results in noisy data.

These products often can be visualized on an agarose gel before sequencing. If they are present, the PCR amplification should be optimized and repeated before sequencing. Use of a nested or semi-nested sequencing primer can also allow good sequence data to be obtained. Alternatively, the PCR product of interest can be purified by agarose gel electrophoresis.

Minimizing Contaminants

There are several ways to minimize contaminants in a PCR amplification:

- PCR optimization (Innis and Gelfand, 1990):
  - Amount of starting DNA
  - Careful primer design
  - Primer concentration
  - Enzyme concentration
  - Magnesium ion (Mg^{2+}) concentration
  - Nucleotide concentration
  - Buffer composition
  - Number of cycles
  - pH
- Manual Hot Start method
- AmpliTaq Gold® DNA Polymerase as an automatic Hot Start
- Limiting dNTPs and primers

All of these methods increase the specificity of the PCR amplification and decrease the amount of contaminants that can interfere with a sequencing reaction.

Preparing PCR Products for Sequencing

Purification

There are several methods for purifying PCR products:

- Column purification
- Ethanol precipitation
- Gel purification

IMPORTANT If more than one PCR product is present, neither column purification nor ethanol precipitation will isolate the desired product. Use gel purification to isolate the desired product or reoptimize the PCR to obtain a single product.

Commercially available products for PCR product purification are listed below:

- Centricon®-100 columns (P/N N930-2119)

  These columns contain an ultrafiltration membrane that separates primers and dNTPs from larger PCR products. However, they may not work as well for short PCR products (<125 bases).

To purify PCR fragments by ultrafiltration:

Step	Action
1	Assemble the Centricon-100 column according to the manufacturer's recommendations.
2	Load 2 mL deionized water onto the column.
3	Add the entire sample to the column.
4	Spin the column at 3000 × *g* in a fixed-angle centrifuge for 10 minutes. **Note** The manufacturer recommends a maximum speed of 1000 × *g*, but 3000 × *g* has worked well in Applied Biosystems. If you are following the manufacturer's guidelines, increase the time to compensate.

To purify PCR fragments by ultrafiltration: *(continued)*

Step	Action
5	Remove the waste receptacle and attach the collection vial.
6	Invert the column and spin it at 270 × *g* for 2 minutes to collect the sample. This should yield approximately 40–60 µL of sample.
7	Add deionized water to bring the purified PCR fragments to the original volume.

- QIAquick PCR Purification Kits (QIAGEN: P/N 28104, 50 reactions; 28106, 250 reactions)

  These kits work well for PCR products ranging from 100 bp–10 kbp.

- QIAquick Gel Extraction Kits (QIAGEN: P/N 28704, 50 reactions; 28706, 250 reactions)

  These kits are used to purify PCR fragments from agarose gels. The kits work well for DNA ranging from 70 bp–10 kbp. Fragments larger than this should be extracted with the QIAEX II Gel Extraction Kits (QIAGEN: P/N 20021, 150 reactions; 20051, 500 reactions).

Refer to the manufacturer's instructions for the procedures.

The recommended DNA quantities for sequencing reactions are shown in Table 3-1 on page 3-17.

Shrimp Alkaline Phosphatase and Exonuclease I Treatment

An alternative to one of the more stringent purification methods listed above is treatment of PCR products with shrimp alkaline phosphatase (SAP) and exonuclease I (Exo I) before sequencing. The SAP/Exo I procedure degrades nucleotides and single-stranded DNA (primers) remaining after PCR (Werle *et al.*, 1994). This procedure is particularly useful in cases where limiting concentrations of primers and nucleotides cannot be used for direct PCR sequencing (see page 3-14).

IMPORTANT This method only works when a single PCR product is obtained.

To degrade PCR primers and nucleotides using SAP/Exo I:

Step	Action
1	For each sample, combine the following: ♦ SAP(1 Unit/µL), 2 µL ♦ Exo I (10 Units/µL), 0.2 µL ♦ Deionized water, 6.0 µL **Note** In general this procedure works well using 0.5 units of each enzyme per microliter of PCR products used. The procedure seems to work equally well with or without the use of SAP buffer, so this has been excluded in this protocol.
2	Add 4.0 µL of PCR product to the above mix.
3	Incubate at 37 °C for 1 hour.
4	Incubate at 72 °C for 15 minutes to inactivate the enzymes.

The PCR product may need dilution before sequencing. Determine the dilution ratio empirically (start with 1:2 and 1:10 dilutions with deionized water).

The recommended DNA quantities for sequencing reactions are shown in Table 3-1 on page 3-17.

Direct PCR Sequencing

PCR protocols that limit amounts of primers and dNTPs allow the product of the reaction to be used for sequencing with no purification. This is usually carried out by setting up the PCR amplification with 5–10 pmol of primers and 20–40 µM dNTPs, so that most of the primers and dNTPs are exhausted during amplification.

If the yield of the desired PCR product is high and the product is specific, *i.e.*, it produces a single band when analyzed by agarose gel electrophoresis, the sample can be diluted before sequencing and will give good results. The dilution ratio depends on the concentration of your PCR product and needs to be determined empirically (start with 1:2 and 1:10 dilutions with deionized water).

When you limit concentrations of primers and dNTPs and dilute the PCR products, the PCR parameters have to be robust. Direct PCR sequencing is most useful in applications where the same target is being amplified and sequenced repeatedly and PCR conditions have been optimized.

Direct PCR sequencing is usually done with dye primer chemistries. With dye terminator chemistries, it is much more critical that the PCR primers be consumed. Excess PCR primers will be extended and labeled by the cycle sequencing reaction, resulting in noisy data.

Direct PCR sequencing does not work for XL PCR because limiting amounts of primers and dNTPs cannot be used. The PCR product should be purified (see page 3-12) or the excess primers and nucleotides should be degraded by SAP/Exo I treatment (see page 3-13).

DNA Template Quality

Using Control DNA

Include a control DNA template as one of the templates in a set of sequencing reactions. The results from the control can help determine whether failed reactions are the result of poor template quality or sequencing reaction failure.

We recommend M13mp18 as a single-stranded control and pGEM®-3Zf(+) as a double-stranded control. All Applied Biosystems DNA sequencing kits provide pGEM control DNA. All dye terminator cycle sequencing kits include a –21 M13 control primer.

Sequencing Standards

The Cycle Sequencing Standards provide an additional control to help in troubleshooting electrophoresis runs. These standards contain lyophilized sequencing reactions that only require resuspension and denaturation before use. There are four standards available:

- Dye Primer Cycle Sequencing Standard (P/N 401920)
- Dye Terminator Cycle Sequencing Standard (P/N 402830)
- dRhodamine Terminator Cycle Sequencing Standard (P/N 4303120)
- BigDye™ Terminator Cycle Sequencing Standard (P/N 4304154)

Poor Template Quality

Poor template quality is the most common cause of sequencing problems. Always follow recommended procedures to prepare templates (see “DNA Template Preparation” on page 3-2).

The following are characteristics of poor quality templates:

- Noisy data or peaks under peaks (see page 7-11)
- No usable sequence data (see Figure 7-9 on page 7-10)
- Weak signal (see Figure 7-10 on page 7-11)

Contamination

Potential contaminants include:

- Proteins
- RNA
- Chromosomal DNA
- Excess PCR primers, dNTPs, enzyme, and buffer components (from a PCR amplification used to generate the sequencing template)
- Residual salts
- Residual organic chemicals, *e.g.*, phenol, chloroform, and ethanol
- Residual detergents

Host Strain Variability

The host strain used for template preparation can impact template quality. One host strain may produce better sequencing results for a specific template than another.

If you plan to use a commercial template preparation kit, contact the vendor for information about host strains that work well with that kit.

A good source of information relating to host strain effects can be found in the *QIAGEN Guide to Template Preparation and DNA Sequencing (2nd edition)*. Contact your local QIAGEN office (http://www.qiagen.com/qiagenww.html) to obtain a copy of this guide.

Determining DNA Quality

The following methods can be used to examine DNA quality:

- Agarose gel electrophoresis

  Purified DNA should run as a single band on an agarose gel.

Note Uncut plasmid DNA can run as three bands: supercoiled, nicked, and linear.

- Spectrophotometry

  The A_{260}/A_{280} ratio should be 1.7–1.9. Smaller ratios usually indicate contamination by protein or organic chemicals.

Agarose gels reveal the presence of contaminating DNAs and RNAs, but not proteins. Spectrophotometry can indicate the presence of protein contamination, but not DNA and RNA contamination. These methods should be used together to get the most information about your DNA template before sequencing.

Note RNA contamination up to 1 µg can be tolerated, but it will affect DNA quantitation greatly.

Neither of these methods shows the presence of contaminating salts that can cause noisy data. If you suspect that your DNA is contaminated with salt, remove the salt before sequencing. The most efficient method for salt removal is ultrafiltration with a Centricon-100 column (see page 3-12). Spin columns and ethanol precipitation can also be used (see page 3-33).

Cleaning Up Dirty Templates

A “dirty” template preparation sometimes can be cleaned up with one of the following methods:

- Purify the DNA by ultrafiltration. Use Centricon-100 Micro-Concentrator columns (see “Preparing PCR Products for Sequencing” on page 3-12).
- Purify by extraction:

Step	Action
1	Extract the DNA twice with 1 volume of chloroform or chloroform:isoamyl alcohol (24:1 v/v).
2	Add 0.16 volumes of 5M NaCl and 1 total volume of 13% PEG.
3	Incubate on ice for 20 minutes, then centrifuge at maximum speed in a microcentrifuge at 2–6 °C for 20 minutes.
4	Rinse the pellet twice with 70% ethanol.
5	Dry the pellet in a vacuum centrifuge for 3–5 minutes or to dryness.

DNA Template Quantity

Quantitating DNA

DNA template quantitation is critical for successful sequencing reactions. The most common way to determine DNA quantity is to measure the absorbance (optical density or O.D.) of a sample at 260 nm in a spectrophotometer.

One O.D. unit is the amount of a substance dissolved in 1.0 mL that gives an absorbance reading of 1.00 in a spectrophotometer with a 1-cm path length. The wavelength is assumed to be 260 nm unless stated otherwise. A_{260} values can be converted into ng/µL using Beer´s Law:

Absorbance (260 nm) = sum of extinction coefficient contributions × cuvette pathlength × concentration

The following formulas are derived from Beer's Law (Ausubel *et al.*, 1998):

- One A_{260} unit of single-stranded DNA contains 33 ng/µL.
- One A_{260} unit of double-stranded DNA contains 50 ng/µL.

Note Absorbance measurements of highly concentrated (O.D.>1.0) or very dilute (O.D.<0.05) DNA samples can be inaccurate.

DNA can also be quantitated by fluorometric analysis employing either Hoechst dye #33258 (Hoefer, Inc., 1993) or Picogreen (Molecular Probes, Inc., 1995).

Amount to Use in Sequencing Reactions

The amount of DNA template used in a sequencing reaction can affect the quality of the data. Too much template makes data appear top heavy with strong peaks at the beginning of the run that fade rapidly. Too little template or primer reduces the signal strength and peak height. In the worst case, the noise level increases so that bases cannot be called. Table 3-1 shows the recommended quantities for each sequencing chemistry.

Table 3-1 Recommended Ranges of DNA Template Quantity for Each Chemistry

	Cycle Sequencing Chemistry				
Template	**Rhodamine Dye Terminator**	**dRhodamine Terminator**	**BigDye Terminator**	**Fluorescein/ Rhodamine Dye Primer**	**BigDye Primer**
PCR product:					
100–200 bp	1–3 ng	1–3 ng	1–3 ng	2–5 ng	2–5 ng
200–500 bp	3–10 ng	3–10 ng	3–10 ng	5–10 ng	5–10 ng
500–1000 bp	5–20 ng	5–20 ng	5–20 ng	10–20 ng	10–20 ng
1000–2000 bp	10–40 ng	10–40 ng	10–40 ng	20–50 ng	20–50 ng
>2000 bp	40–100 ng	40–100 ng	40–100 ng	50–150 ng	50–150 ng
single-stranded	100–250 ng	50–100 ng	50–100 ng	150–300 ng	150–400 ng
double-stranded	200–500 ng	200–500 ng	200–500 ng	300–600 ng	200–800 ng
cosmid, BAC	0.5–2.0 µg	not recommended	0.5–1.0 µg	0.5–2.0 µg	0.5–1.0 µg
bacterial genomic DNA	not recommended		2–3 µg	not recommended	

Primer Design and Quantitation

Overview

The choice of primer sequence, method of primer synthesis, and approach to primer purification can have a significant effect on the quality of the sequencing data obtained in dye terminator cycle sequencing reactions. Dye primer cycle sequencing kits include dye-labeled primers that are already optimized and quantitated.

Some of the recommendations given here are based on information that is general knowledge, while others are based on practical experience gained by scientists at Applied Biosystems.

Primer Design

The following recommendations are provided to help optimize primer selection:

- Use Primer Express™ software (P/N 402089) for primer design.

  Primer Express software is useful in identifying potential secondary structure problems, calculating melting temperature (T_m) more accurately, and determining if a secondary hybridization site exists on the target DNA.
- Primers should be at least 18 bases long to ensure good hybridization.
- Avoid runs of an identical nucleotide, especially runs of four or more Gs.
- Keep the G-C content in the range 30–80%, preferably 50–55%.
- For cycle sequencing, primers with T_m>45 °C produce better results than primers with lower T_m using our recommended thermal cycling parameters.
- For primers with a G-C content less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the T_m>45 °C.
- Use of primers longer than 18 bases also minimizes the chance of having a secondary hybridization site on the target DNA.
- Avoid primers that can hybridize to form dimers.
- Avoid palindromes because they can form secondary structures.
- The primer should be as pure as possible, preferably purified by HPLC.

Estimating Melting Temperature

The following formula can be used for a rough estimate of melting temperature:

T_m = (number of A + T residues) × 2 °C + (number of G + C residues) × 4 °C

Primer Quantitation

The following formula, which is derived from Beer's Law, converts A_{260} readings into pmol/µL concentrations:

$$C \text{ (pmol/µL or µM)} = (A_{260} \times 100)/(1.54n_A + 0.75n_C + 1.17n_G + 0.92n_T)$$

where:

C = concentration

n_X = number of residues of base x in the oligonucleotide

Oligonucleotide Molecular Weights

Molecular weight of a DNA oligonucleotide (sodium salt, pH≥7):

$$MW = (N_A \times 335.2) + (N_C \times 311.2) + (N_G \times 351.2) + (N_T \times 326.2) + P$$

where:

N_X = number of residues of base x in the oligonucleotide

P = –101.0 for dephosphorylated oligonucleotides, 40.0 for phosphorylated oligonucleotides

Primer Problems and Possible Causes

Table 3-2 Primer Problems and Possible Causes

Problems	Possible Causes
Poor priming resulting in weak or no signal	Melting temperature is too low due to low GC content and/or short primer length
	Secondary structure of the primer, particularly at the 3´ end
	Secondary structure of the template in the region of hybridization
	Incorrect primer concentration
	Priming site not present
Adequate signal strength with noisy data	Secondary hybridization site, which results in many extra peaks
	Impure primer. You may see a shadow sequence of N–1.

Custom Oligonucleotides

You can obtain custom primers from the Applied Biosystems Custom Oligonucleotide Synthesis Service:

- Phone: (800) 345-5224
- E-mail: support@appliedbiosystems.com
- Online: www.appliedbiosystems.com/techsupport

Reagent and Equipment Considerations

Reagent Handling and Reaction Storage

The freshest reagents are likely to perform the best. The following methods are recommended for guaranteeing reagent freshness.

- Store reagents at –15 to –25 °C when not in use, and thaw completely at room temperature or in an ice bath (do not heat) before use.

Note Do not use a frost-free freezer. The automatic cycling of the temperature for defrosting can damage reagents, particularly enzymes.

- Avoid excess (more than ten) freeze-thaw cycles. Aliquot reagents in smaller amounts if necessary.
- Shield reagents and sequencing reactions from light. Fluorescent dyes are susceptible to bleaching.
- If you would like to store sequencing reactions for future use, purify and dry them. Store the reactions at –15 to –25 °C.

Reaction Tubes

The type of tube required depends on the type of thermal cycler used. For the DNA Thermal Cycler (TC1) and DNA Thermal Cycler 480, use 0.5-mL GeneAmp® Thin-Walled PCR tubes. For the GeneAmp PCR Systems 9700, 9600, and 2400, use 0.2-mL MicroAmp® PCR tubes. If using the CATALYST™ 800 Molecular Biology LabStation or ABI PRISM® 877 Integrated Thermal Cycler, refer to your instrument user's manual for reaction tube selection.

Thermal Cyclers

The type and performance of the thermal cycler used to prepare sequencing reactions can affect the quality of the reactions. Ensure that the thermal cycler is calibrated regularly by the manufacturer and that ramping rates are 1 °C/second.

Preparing Cycle Sequencing Reactions

Rhodamine Dye Terminators

The procedure given here is for the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kits. Refer to the *ABI PRISM Dye Terminator Cycle Sequencing Core Kit Protocol* (P/N 402116) for information on preparing reactions with the core kits.

Step	Action	
1	For each reaction, add the following reagents to a separate tube:	
	Reagent	**Quantity**
	Terminator Ready Reaction Mix	8.0 µL
	Template single-stranded DNA double-stranded DNA PCR product	– 100–250 ng 200–500 ng 1–100 ng (depending on size, see Table 3-1 on page 3-17)
	Primer	3.2 pmol
	Deionized water	q.s.
	Total Volume	20 µL
2	Mix well and spin briefly.	
3	**If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:**	
	Overlay the reaction mixture with 40 µL of light mineral oil.	

The cycle sequencing procedures for rhodamine dye terminators start on page 3-27.

dRhodamine Terminators

Step	Action	
1	For each reaction, add the following reagents to a separate tube:	
	Reagent	**Quantity**
	Terminator Ready Reaction Mix	8.0 µL
	Template single-stranded DNA double-stranded DNA PCR product	– 50–100 ng 200–500 ng 1–100 ng (depending on size, see Table 3-1 on page 3-17)
	Primer	3.2 pmol
	Deionized water	q.s.
	Total Volume	20 µL
2	Mix well and spin briefly.	
3	**If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:**	
	Overlay the reaction mixture with 40 µL of light mineral oil.	

The cycle sequencing procedures for dRhodamine terminators start on page 3-27.

BigDye Terminators

The flexibility of the BigDye terminators allows three options for cycle sequencing:

Reaction Type	Template	Cycle
1X	♦ PCR product ♦ plasmid ♦ M13	standard
0.5X	♦ PCR product ♦ plasmid ♦ M13	standard
High-sensitivity (2X)	♦ Large DNA templates ♦ bacterial genomic DNA	modified

The cycle sequencing procedures for BigDye terminators start on page 3-27.

1X Reactions

Step	Action	
1	For each reaction, add the following reagents to a separate tube:	
	Reagent	**Quantity**
	Terminator Ready Reaction Mix	8.0 µL
	Template single-stranded DNA double-stranded DNA PCR product DNA	– 50–100 ng 200–500 ng 1–100 ng (depending on size, see Table 3-1 on page 3-17)
	Primer	3.2 pmol
	Deionized water	q.s.
	Total Volume	20 µL
2	Mix well and spin briefly.	
3	**If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:**	
	Overlay the reaction mixture with 40 µL of light mineral oil.	

0.5X Reactions

Dilute 5X Sequencing Buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0—P/N 4305605, 600 reactions; 4305603, 5400 reactions) with an equal volume of deionized water to 2.5X for use in this procedure.

Step	Action	
1	For each reaction, add the following reagents to a separate tube:	
	Reagent	**Quantity**
	Terminator Ready Reaction Mix	4.0 µL
	2.5X Sequencing Buffer	4.0 µL
	Template single-stranded DNA double-stranded DNA PCR product DNA	– 50–100 ng 200–500 ng 1–100 ng (depending on size, see Table 3-1 on page 3-17)
	Primer	3.2 pmol
	Deionized water	q.s.
	Total Volume	20 µL
2	Mix well and spin briefly.	
3	**If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:**	
	Overlay the reaction mixture with 40 µL of light mineral oil.	

High-Sensitivity (2X) Reactions for BACs, PACs, YACs, and Cosmids

Step	Action	
1	For each reaction, add the following reagents to a separate tube:	
	Reagent	**Quantity**
	Terminator Ready Reaction Mix	16 µL
	DNA Template	0.5–1.0 µg
	Primer	5–10 pmol
	Deionized water	q.s.
	Total Volume	40 µL
2	Mix well and spin briefly.	

Note These high-sensitivity reactions have been optimized on the GeneAmp PCR System 9600 or 9700 (in 9600 emulation mode). The protocols would need to be reoptimized for use on other thermal cyclers.

The cycle sequencing procedure is on page 3-28.

High-Sensitivity (2X) Reactions for Bacterial Genomic DNA

Step	Action	
1	For each reaction, add the following reagents to a separate tube:	
	Reagent	**Quantity**
	Terminator Ready Reaction Mix	16 µL
	DNA Template[a]	2–3 µg
	Primer	6–13 pmol
	Deionized water	q.s.
	Total Volume	40 µL
2	Mix well and spin briefly.	

a. Shearing the DNA by passing it seven times through a 21-gauge, 1-inch long needle can improve signals.

Note These high-sensitivity reactions have been optimized on the GeneAmp PCR System 9600 or 9700 (in 9600 emulation mode). The protocols would need to be reoptimized for use on other thermal cyclers.

The cycle sequencing procedure is on page 3-29.

Fluorescein/ Rhodamine Dye Primers

The procedure given here is for the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kits. Refer to the *ABI PRISM Dye Primer Cycle Sequencing Core Kit Protocol* (P/N 402114) for information on preparing reactions with the core kits.

Step	Action				
1	Aliquot the following reagents into four PCR tubes:				
	Reagent	**A** (µL)	**C** (µL)	**G** (µL)	**T** (µL)
	Ready Reaction Premix	4	4	8	8
	DNA Template (see Table 3-1 on page 3-17 for quantity)	1	1	2	2
	Total Volume	5	5	10	10
2	**If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:**				
	a. Add 20 µL of light mineral oil. b. Spin to layer the oil over the aqueous reaction.				

The cycle sequencing procedures for fluorescein/rhodamine dye primers start on page 3-29.

BigDye Primers

The flexibility of the BigDye™ primers allows three options for cycle sequencing:

Reaction Type	Template	Cycle
1X	♦ PCR product ♦ plasmid ♦ M13	standard
0.5X	♦ PCR product ♦ plasmid ♦ M13	standard
High-sensitivity (2X)	♦ Large DNA template containing –21 M13 and/or M13 Reverse priming site	modified

IMPORTANT Prepare separate tubes for each of the four reactions (A, C, G, and T).

The cycle sequencing procedures for BigDye primers start on page 3-29.

1X Reactions

Step	Action				
1	Aliquot the following reagents into four PCR tubes:				
	Reagent	**A** (µL)	**C** (µL)	**G** (µL)	**T** (µL)
	Ready Reaction Premix	4	4	4	4
	DNA Template (see Table 3-1 on page 3-17 for quantity)	1	1	1	1
	Total Volume	5	5	5	5
2	**If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:**				
	a. Add 20 µL of light mineral oil. b. Spin to layer the oil over the aqueous reaction.				

0.5X Reactions

Dilute 5X Sequencing Buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0—P/N 4305605, 600 reactions; 4305603, 5400 reactions) with four parts deionized water to 1X for use in this procedure.

Step	Action				
1	Dilute each Ready Reaction Premix (A, C, G, T) 1:1 with 1X Sequencing Buffer in a separate tube (*e.g.*, 2 µL of A Mix and 2 µL of 1X Sequencing Buffer).				
2	Aliquot the following reagents into four PCR tubes for each DNA template:				
	Reagent	**A** (µL)	**C** (µL)	**G** (µL)	**T** (µL)
	Diluted Ready Reaction Premix	4	4	4	4
	DNA Template (see Table 3-1 on page 3-17 for quantity)	1	1	1	1
	Total Volume	5	5	5	5
3	**If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:**				
	a. Add 20 µL of light mineral oil. b. Spin to layer the oil over the aqueous reaction.				

High-Sensitivity (2X) Reactions

Aliquot the following reagents into four PCR tubes:

Reagent	A (µL)	C (µL)	G (µL)	T (µL)
Ready Reaction Premix	8	8	8	8
DNA Template (see Table 3-1 on page 3-17 for quantity)	2	2	2	2
Total Volume	10	10	10	10

Note These high-sensitivity reactions have been optimized on the GeneAmp PCR System 9600 or 9700 (in 9600 emulation mode). The protocols would need to be reoptimized for use on other thermal cyclers.

The cycle sequencing procedure is on page 3-30.

Cycle Sequencing

Overview

These protocols have been optimized for all Applied Biosystems thermal cyclers, including the DNA Thermal Cycler (TC1), the DNA Thermal Cycler 480, the CATALYST 800 Molecular Biology LabStation, the ABI PRISM 877 Integrated Thermal Cycler, and the GeneAmp PCR Systems 9600, 2400, and 9700 in 9600 emulation mode. The protocols contained in this section should work for all seven instruments.

If you use a thermal cycler not manufactured by Applied Biosystems, you may need to optimize thermal cycling conditions. Ramping time is very important. If the thermal ramping time is too fast (>1 °C/sec), poor (noisy) data may result.

Dye Terminator Chemistries

These protocols, except for sequencing BAC DNA and other large templates, are used for the rhodamine dye terminator, dRhodamine terminator, and BigDye terminator chemistries.

These conditions work for a variety of templates and primers. However, if necessary, these parameters can be changed to suit particular situations, including the following:

- For short PCR products, you can use reduced numbers of cycles (*e.g.*, 20 cycles for a 300-bp or smaller fragment).
- If the T_m of a primer is >60 °C, the annealing step can be eliminated.
- If the T_m of a primer is <50 °C, increase the annealing time to 30 seconds or decrease the annealing temperature to 48 °C.
- For templates with high GC content (>70%), heat the tubes at 98 °C for 5 minutes before cycling to help denature the template.

GeneAmp 9700 (in 9600 Emulation Mode), 9600, or 2400

Step	Action
1	Place the tubes in a thermal cycler and set the volume to 20 µL.
2	Repeat the following for 25 cycles: ♦ Rapid thermal ramp[a] to 96 °C ♦ 96 °C for 10 sec. ♦ Rapid thermal ramp to 50 °C ♦ 50 °C for 5 sec. ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 4 min.
3	Rapid thermal ramp to 4 °C and hold until ready to purify.
4	Spin down the contents of the tubes in a microcentrifuge.

a. Rapid thermal ramp is 1 °C/sec.

DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480

Step	Action
1	Place the tubes in a thermal cycler and set the volume to 20 µL.
2	Repeat the following for 25 cycles: ♦ Rapid thermal ramp[a] to 96 °C ♦ 96 °C for 30 sec. ♦ Rapid thermal ramp to 50 °C ♦ 50 °C for 15 sec. ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 4 min.
3	Rapid thermal ramp to 4 °C and hold until ready to purify.
4	Spin down the contents of the tubes in a microcentrifuge.

a. Rapid thermal ramp is 1 °C/sec.

BACs, PACs, YACs, and Cosmids on the GeneAmp 9700 (in 9600 Emulation Mode), 9600, or 2400

Note This protocol is for use only with the BigDye terminator kits.

Step	Action
1	Place the tubes in a thermal cycler and set the volume to 40 µL.
2	Heat the tubes at 95 °C for 5 minutes.
3	Repeat the following for 30 cycles:[a] ♦ Rapid thermal ramp[b] to 95 °C ♦ 95 °C for 30 sec. ♦ Rapid thermal ramp to 50–55 °C (depending on template) ♦ 50–55 °C for 10 sec. ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 4 min.
4	Rapid thermal ramp to 4 °C and hold until ready to purify.
5	Spin down the contents of the tubes in a microcentrifuge.

a. Some laboratories have found that increasing the number of cycles gives better results.

b. Rapid thermal ramp is 1 °C/sec.

Bacterial Genomic DNA on the GeneAmp 9700 (in 9600 Emulation Mode), 9600, or 2400

Note This protocol is for use only with the BigDye terminator kits.

Step	Action
1	Place the tubes in a thermal cycler and set the volume to 40 µL.
2	Heat the tubes at 95 °C for 5 minutes.
3	Repeat the following for 45 cycles: ♦ Rapid thermal ramp[a] to 95 °C ♦ 95 °C for 30 sec. ♦ Rapid thermal ramp to 50–55 °C (depending on template) ♦ 55 °C for 20 sec. ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 4 min.
4	Rapid thermal ramp to 4 °C and hold until ready to purify.
5	Spin down the contents of the tubes in a microcentrifuge.

a. Rapid thermal ramp is 1 °C/sec.

Dye Primer Chemistries

These protocols, except for BAC DNA sequencing, are used for the fluorescein/rhodamine dye primer and BigDye primer chemistries.

GeneAmp 9700 (in 9600 Emulation Mode), 9600, or 2400

Step	Action
1	Place the tubes in a thermal cycler and set the volume to 5 µL.
2	Repeat the following for 15 cycles: ♦ Rapid thermal ramp[a] to 96 °C ♦ 96 °C for 10 sec. ♦ Rapid thermal ramp to 55 °C ♦ 55 °C for 5 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
3	Repeat the following for 15 cycles: ♦ Rapid thermal ramp to 96 °C ♦ 96 °C for 10 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
4	Rapid thermal ramp to 4 °C and hold until ready to pool and precipitate.

a. Rapid thermal ramp is 1 °C/sec.

TC1 or DNA Thermal Cycler 480

Step	Action
1	Place the tubes in a thermal cycler preheated to 95 °C.
2	Repeat the following for 15 cycles: ♦ Rapid thermal ramp[a] to 95 °C ♦ 95 °C for 30 sec. ♦ Rapid thermal ramp to 55 °C ♦ 55 °C for 30 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
3	Repeat the following for 15 cycles: ♦ Rapid thermal ramp to 95 °C ♦ 95 °C for 30 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
4	Rapid thermal ramp to 4 °C and hold until ready to pool and precipitate.

a. Rapid thermal ramp is 1 °C/sec.

BAC DNA on the GeneAmp 9700 (in 9600 Emulation Mode) or 9600

Note This protocol is for use only with the BigDye primer kits.

Step	Action
1	Place the tubes in a thermal cycler, set the volume at 10 µL, and begin thermal cycling with the following parameters: ♦ Rapid thermal ramp[a] to 95 °C ♦ 95 °C for 5 min.
2	Repeat the following for 20 cycles: ♦ Rapid thermal ramp to 95 °C ♦ 95 °C for 30 sec. ♦ Rapid thermal ramp to 50 °C ♦ 50 °C for 15 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
3	Repeat the following for 15 cycles: ♦ Rapid thermal ramp to 95 °C ♦ 95 °C for 30 sec. ♦ Rapid thermal ramp to 70 °C ♦ 70 °C for 1 min.
4	Rapid thermal ramp to 4 °C and hold until ready to pool and precipitate.

a. Rapid thermal ramp is 1 °C/sec.

Cycle Sequencing on the CATALYST 800

Templates that have been prepared as described in this chapter should be suitable for use on the CATALYST 800 Molecular Biology LabStation using LabStation 3.0 protocols. Follow the protocols in the Turbo Appendix of the *CATALYST 800 Molecular Biology LabStation User's Manual* (P/N 903939) to set up your reactions.

Dye Terminator Sequencing Options

Terminator Sequencing has two options:

- Using a reaction premix containing the sequencing primer or premixing template with primer in the sample tube
- Combining reaction cocktail (lacking primers), water, and primer from one tube and template from another tube

  This eliminates the requirement for premixing samples and primers.

Dye Primer Sequencing Options

Predefined temperature profiles are provided for the following:

- Double-Stranded Forward (Universal) Primer
- Double-Stranded Reverse (Universal) Primer
- Single-Stranded Forward Primer
- Quick Cycle

These are chosen during the pre-run dialogue, and can be edited to make custom profiles.

IMPORTANT Load only the reagents that you plan to use. Do not store kit reagents on the worksurface.

Cycle Sequencing on the ABI PRISM 877 ITC

Dye Terminator Sequencing Options

Predefined temperature profiles are provided for the following on the ABI PRISM 877 Integrated Thermal Cycler:

- Terminator Sequencing uses a reaction premix containing the sequencing primer, or else requires premixing template with primer in the sample tube.
- Terminator Automix Sequencing combines reaction cocktail (lacking primers), water, primer from one tube, and template from another tube. This eliminates the requirement for premixing of samples and primers.

The profile is chosen on the Chemistry page of the Sequencing Notebook and can be edited to make custom profiles. Refer to Chapter 4, "Using the ABI PRISM 877 Software," in the ABI PRISM 877 *Integrated Thermal Cycler User's Manual* (P/N 904414).

Dye Primer Sequencing Options

Predefined temperature profiles are provided for the following on the ABI PRISM 877 Integrated Thermal Cycler:

- Double-Stranded Forward (Universal) Primer
- Double-Stranded Reverse (Universal) Primer
- Single-Stranded Forward Primer
- Quick Cycle

These are chosen on the Chemistry page of the Sequencing Notebook, and can be edited to make custom profiles. Refer to Chapter 4, "Using the ABI PRISM 877 Software," in the ABI PRISM 877 *Integrated Thermal Cycler User's Manual* for instructions on editing temperature profiles.

IMPORTANT Load only the reagents that you plan to use. Do not store kit reagents on the worksurface.

Preparing Extension Products for Electrophoresis

Overview

Preparation of extension products for electrophoresis will vary depending on the cycle sequencing chemistry used.

Dye Terminator Chemistries

Unincorporated dye terminators must be removed before the samples can be analyzed by electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with basecalling.

Several protocols for each sequencing chemistry are presented to offer a choice of reagents and process. We recommend performing controlled reactions with each method to determine the one that works best for you.

- Precipitation methods are cheaper and faster, but if performed poorly can leave unincorporated dye-labeled terminators that can obscure data at the beginning of the sequence.

  Refer to the *Precipitation Methods to Remove Residual Dye Terminators from Sequencing Reactions User Bulletin* (P/N 4304655). This document can be obtained from the Applied Biosystems WWW site (www.appliedbiosystems.com/techsupport).

- The spin column and 96-well plate procedures remove all excess terminators if performed correctly, but are more costly than precipitation methods.

Dye Primer Chemistries

The standard procedure is ethanol precipitation, which concentrates the sample. An Express Load option is also available for BigDye primers (see page 3-49).

Table 3-3 Recommended Methods for Preparing Extension Products for Electrophoresis

Chemistry	Recommended Methods	See Page
Rhodamine Dye Terminator and dRhodamine Terminator	Spin Column Purification	3-34
	96-Well Plate Purification Protocol	3-35
	Ethanol/Sodium Acetate Precipitation	3-41
	Ethanol/$MgCl_2$ Precipitation	3-43
	Shrimp Alkaline Phosphatase Digestion	3-45
BigDye Terminator	Spin Column Purification	3-34
	96-Well Plate Purification Protocol	3-35
	Isopropanol Precipitation	3-36
	Ethanol Precipitation for BigDye Terminators	3-38
	Ethanol/Sodium Acetate Precipitation	3-41
Fluorescein/ Rhodamine Dye Primer	Ethanol Precipitation for Fluorescein/Rhodamine Dye Primers	3-46
BigDye Primer	Ethanol Precipitation for BigDye Primers	3-47
	Express Load Option for BigDye Primers Run on 36-Lane Gels	3-49

Removing Unincorporated Dye Terminators

Spin Column Purification

We recommend Centri-Sep™ spin columns from Princeton Separations (P/N CS-901).

IMPORTANT For the BigDye terminators, hydrate the column for 2 hours.

Tips for optimizing spin column purification:

- Use one column for each sample.
- Do not process more columns than you can handle conveniently at one time.
- Load the sample in the center of the column bed. Make sure that the sample does not touch the sides of the column and that the pipet tip does not touch the gel surface.

  If samples are not properly loaded, peaks from unincorporated dye terminators can result (see Figure 7-36 on page 7-29).
- Spin the column at 325–730 × *g* for best results. Use the following formula to calculate the best speed for your centrifuge:

  $g = 11.18 \times r \times (\text{rpm}/1000)^2$

  where:

  g = relative centrifugal force

  rpm = revolutions per minute

  r = radius of the rotor in cm
- Do not spin for more than 2 minutes.
- Perform the entire procedure without interruption to ensure optimal results. Do not allow the column to dry out.

To perform spin column purification:

Step	Action
1	Gently tap the column to cause the gel material to settle to the bottom of the column.
2	Remove the upper end cap and add 0.8 mL of deionized water.
3	Replace the upper end cap and vortex or invert the column a few times to mix the water and gel material.
4	Allow the gel to hydrate at room temperature for 30 minutes (at least 2 hours for BigDye terminators). **Note** Hydrated columns can be stored for a few days at 2–6 °C. Longer storage in water is not recommended. Allow columns that have been stored at 2–6 °C to warm to room temperature before use.
5	Remove any air bubbles by inverting or tapping the column and allowing the gel to settle.
6	Remove the upper end cap first, then remove the bottom cap. Allow the column to drain completely by gravity. **Note** If flow does not begin immediately, apply gentle pressure to the column with a pipette bulb.
7	Insert the column into the wash tube provided.
8	Spin the column in a microcentrifuge at 730 × *g* for 2 minutes to remove the interstitial fluid.

To perform spin column purification: *(continued)*

Step	Action
9	Remove the column from the wash tube and insert it into a sample collection tube (*e.g.*, a 1.5-mL microcentrifuge tube).
10	Remove the extension reaction mixture from its tube and load it carefully onto the center of the gel material. **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 1 on page 3-37.
11	Spin the column in a microcentrifuge at 730 × *g* for 2 minutes. **Note** If using a centrifuge with a fixed-angle rotor, place the column in the same orientation as it was in for the first spin. This is important because the surface of the gel will be at an angle in the column after the first spin.
12	Discard the column. The sample is in the sample collection tube.
13	Dry the sample in a vacuum centrifuge for 10–15 minutes, or until dry. Do not over-dry.

96-Well Plate Purification Protocol

For large-scale procedures, you can use the following commercially available 96-well plates:

- 96-Well Gel Filtration Block (Edge Biosystems, P/N 21520, 192 reactions; 91751, 960 reactions)
- Multiscreen 96-Well Filter Plates (Millipore, P/N MADYEKIT1)

Refer to the manufacturer's instructions for the procedures.

Isopropanol Precipitation

Note These procedures are for use only with the BigDye terminators.

Precipitation in 96-Well MicroAmp Trays

Reagents and equipment required:

- Variable speed table-top centrifuge with microtiter plate tray, capable of reaching at least 1400 × *g*
- Strip caps or adhesive-backed aluminum foil tape (3M Scotch Tape 425-3)[1]
- 75% Isopropanol (2-propanol) or 100% isopropanol (anhydrous) at room temperature

Note This procedure does not use salt.

To precipitate extension products in MicroAmp Trays:

Step	Action
1	Remove the MicroAmp Tray from the thermal cycler. Remove the caps from each tube.
2	Add one of the following: ♦ 80 µL of 75% isopropanol or ♦ 20 µL of deionized water and 60 µL of 100% isopropanol The final isopropanol concentration should be 60 ± 5%.
3	Seal the tubes with strip caps or by applying a piece of 3M Scotch Tape 425-3 adhesive-backed aluminum foil tape. Press the foil onto the tubes to prevent any leakage.
4	Invert the tray a few times to mix.
5	Leave the tray at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.
6	Place the tray in a table-top centrifuge with tube-tray adaptor and spin it at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: ♦ 1400–2000 × *g*: 45 minutes ♦ 2000–3000 × *g*: 30 minutes **Note** A MicroAmp tube in a MicroAmp Tray can withstand 3000 × *g* for 30 minutes. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
7	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the tray onto a paper towel folded to the size of the tray.
8	Place the inverted tray with the towel into the table-top centrifuge and spin at 700 × *g* for 1 minute.

1. Contact 3M in the USA at (800) 364-3577 for your local 3M representative. Use of other tapes may result in leakage or contamination of the sample.

To precipitate extension products in MicroAmp Trays: *(continued)*

Step	Action
9	Remove the tray and discard the paper towel. **Note** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.

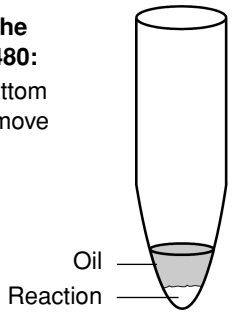
Precipitation in Microcentrifuge Tubes

Reagents and equipment required for this method:

- 1.5-mL microcentrifuge tubes
- Benchtop microcentrifuge, capable of reaching at least 14000 × *g*
- Vacuum centrifuge
- 75% Isopropanol (2-propanol) or 100% isopropanol (anhydrous) at room temperature

Note This procedure does not use salt.

To precipitate extension products in microcentrifuge tubes:

Step	Action
1	Pipet the entire contents of each extension reaction into a 1.5-mL microcentrifuge tube. **To remove reactions run on the TC1 or DNA Thermal Cycler 480:** Place the pipette tip into the bottom of the reaction and carefully remove the reaction from the oil.  **IMPORTANT** Transfer as little oil as possible.
2	Add one of the following: ♦ 80 µL of 75% isopropanol or ♦ 20 µL of deionized water and 60 µL of 100% isopropanol The final isopropanol concentration should be 60 ± 5%.
3	Close the tubes and vortex briefly.
4	Leave the tubes at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.
5	Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 20 minutes at maximum speed. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.

To precipitate extension products in microcentrifuge tubes: *(continued)*

Step	Action
6	Carefully aspirate the supernatants with a separate pipette tip for each sample and discard. Pellets may or may not be visible. **IMPORTANT** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.
7	Add 250 µL of 75% isopropanol to the tubes and vortex them briefly.
8	Place the tubes in the microcentrifuge in the same orientation as in step 5 and spin for 5 minutes at maximum speed.
9	Aspirate the supernatants carefully as in step 6.
10	Dry the samples in a vacuum centrifuge for 10–15 minutes or to dryness. (Alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90 °C for 1 minute.)

Ethanol Precipitation for BigDye Terminators

Note These procedures are for use with BigDye terminators only.

With ethanol precipitation, traces of unincorporated terminators may be seen at the beginning of the sequence data (up to base 40), but this is usually minimal. Some loss in the recovery of the smallest fragments may also be observed.

IMPORTANT Where 95% ethanol is recommended in precipitation protocols, purchase non-denatured ethanol at this concentration rather than absolute (100%) ethanol. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some protocols.

Precipitation in 96-Well MicroAmp Trays

Reagents and equipment required:

- Variable speed table-top centrifuge with microtiter plate tray, capable of reaching at least 1400 × *g*
- Strip caps or adhesive-backed aluminum foil tape (3M Scotch Tape 425-3)[1]
- 95% Ethanol (ACS reagent grade), non-denatured

Note This procedure does not use salt.

To precipitate extension products in MicroAmp Trays:

Step	Action
1	Remove the MicroAmp Tray from the thermal cycler. Remove the caps from each tube.
2	Add the following: ♦ 16 µL of deionized water ♦ 64 µL of non-denatured 95% ethanol The final ethanol concentration should be 60 ± 3%.

1. Contact 3M in the USA at (800) 364-3577 for your local 3M representative. Use of other tapes may result in leakage or contamination of the sample.

To precipitate extension products in MicroAmp Trays: *(continued)*

Step	Action
3	Seal the tubes with strip caps or by applying a piece of 3M Scotch Tape 425-3 adhesive-backed aluminum foil tape. Press the foil onto the tubes to prevent any leakage.
4	Invert the tray a few times to mix.
5	Leave the tray at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.
6	Place the tray in a table-top centrifuge with tube-tray adaptor and spin it at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: ♦ 1400–2000 × *g*: 45 minutes ♦ 2000–3000 × *g*: 30 minutes **Note** A MicroAmp tube in a MicroAmp Tray can withstand 3000 × *g* for 30 minutes. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
7	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the tray onto a paper towel folded to the size of the tray.
8	Place the inverted tray with the towel into the table-top centrifuge and spin at 700 × *g* for 1 minute.
9	Remove the tray and discard the paper towel. **Note** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.

Precipitation in Microcentrifuge Tubes

Reagents and equipment required for this method:

- 1.5-mL microcentrifuge tubes
- Benchtop microcentrifuge, capable of reaching at least 14000 × *g*
- Vacuum centrifuge
- 95% Ethanol (ACS reagent grade), non-denatured

Note This procedure does not use salt.

To precipitate extension products in microcentrifuge tubes:

Step	Action
1	Pipet the entire contents of each extension reaction into a 1.5-mL microcentrifuge tube. **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 1 on page 3-37.
2	Add the following: ♦ 16 µL of deionized water ♦ 64 µL of non-denatured 95% ethanol The final ethanol concentration should be 60 ± 3%.

To precipitate extension products in microcentrifuge tubes: *(continued)*

Step	Action
3	Close the tubes and vortex briefly.
4	Leave the tubes at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.
5	Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 20 minutes at maximum speed. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
6	Carefully aspirate the supernatants with a separate pipette tip for each sample and discard. Pellets may or may not be visible. **IMPORTANT** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.
7	Add 250 µL of 70% ethanol to the tubes and vortex them briefly.
8	Place the tubes in the microcentrifuge in the same orientation as in step 5 and spin for 10 minutes at maximum speed.
9	Aspirate the supernatants carefully as in step 6.
10	Dry the samples in a vacuum centrifuge for 10–15 minutes or to dryness. (Alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90 °C for 1 minute.)

Ethanol/Sodium Acetate Precipitation

IMPORTANT Use non-denatured 95% ethanol rather than absolute (100%) ethanol. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some protocols.

Precipitation in 96-Well MicroAmp Trays

Reagents and equipment required:

- Variable speed table-top centrifuge with microtiter plate tray, capable of reaching at least 1400 × *g*
- Strip caps or adhesive-backed aluminum foil tape (3M Scotch Tape 425-3)[1]
- Sodium acetate (NaOAc), 3 M, pH 4.6 (P/N 400320)
- 95% Ethanol (ACS reagent grade), non-denatured

To precipitate extension products in MicroAmp Trays:

Step	Action
1	Remove the MicroAmp Tray from the thermal cycler. Remove the caps from each tube.
2	Add the following: ♦ 2.0 µL of 3 M sodium acetate (NaOAc), pH 4.6 ♦ 50 µL of 95% ethanol (EtOH) The final ethanol concentration should be 65%.
3	Seal the tubes with strip caps or by applying a piece of 3M Scotch Tape 425-3 adhesive-backed aluminum foil tape. Press the foil onto the tubes to prevent any leakage.
4	Invert the tray a few times to mix.
5	Leave the tray at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.
6	Place the tray in a table-top centrifuge with tube-tray adaptor and spin it at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: ♦ 1400–2000 × *g*: 45 minutes ♦ 2000–3000 × *g*: 30 minutes **Note** A MicroAmp tube in a MicroAmp Tray can withstand 3000 × *g* for 30 minutes. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
7	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the tray onto a paper towel folded to the size of the tray.
8	Place the inverted tray with the towel into the table-top centrifuge and spin at 700 × *g* for 1 minute.
9	Add 150 µL of 70% ethanol to each pellet.

1. Contact 3M in the USA at (800) 364-3577 for your local 3M representative. Use of other tapes may result in leakage or contamination of the sample.

To precipitate extension products in MicroAmp Trays: *(continued)*

Step	Action
10	Cap or seal the tubes, then invert the tray a few times to mix.
11	Spin the tray for 10 minutes at maximum speed.
12	Repeat steps 7 and 8.
13	Remove the tray and discard the paper towel. **Note** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.

Precipitation in Microcentrifuge Tubes

Reagents and equipment required:

- 1.5-mL microcentrifuge tubes
- Benchtop microcentrifuge, capable of reaching at least 14000 × *g*
- Vacuum centrifuge
- Sodium acetate (NaOAc), 3 M, pH 4.6 (P/N 400320)
- 95% Ethanol (ACS reagent grade), non-denatured

Step	Action
1	For each sequencing reaction, prepare a 1.5-mL microcentrifuge tube containing the following: ♦ 2.0 µL of 3 M sodium acetate (NaOAc), pH 4.6 ♦ 50 µL of 95% ethanol (EtOH) **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 1 on page 3-37.
2	Pipet the entire contents of each extension reaction into a tube of sodium acetate/ethanol mixture. Mix thoroughly.
3	Vortex the tubes and leave at room temperature for 15 minutes to precipitate the extension products. Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.
4	Spin the tubes in a microcentrifuge for 20 minutes at maximum speed.
5	Carefully aspirate the supernatant with a pipette tip and discard. **IMPORTANT** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.
6	Rinse the pellet with 250 µL of 70% ethanol.
7	Vortex briefly.
8	Spin for 5 minutes in a microcentrifuge at maximum speed. Again, carefully aspirate the supernatant and discard.
9	Dry the pellet in a vacuum centrifuge for 10–15 minutes, or until dry. Do not over-dry. (Alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90 °C for 1 minute.)

Ethanol/$MgCl_2$ Precipitation

Note These procedures are for use only with the rhodamine dye terminator and dRhodamine terminator chemistries.

These procedures require 70% ethanol (EtOH) containing 0.5 mM $MgCl_2$. This reagent can be prepared *in situ* or as a stock solution.

IMPORTANT Use non-denatured 95% ethanol rather than absolute (100%) ethanol to prepare 70% ethanol solutions. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some protocols.

To prepare 70% EtOH/0.5 mM $MgCl_2$ stock solution:

Step	Action
1	Combine the following in a 1.5-mL microcentrifuge tube: ♦ 1 mL 70% EtOH ♦ 1 µL 0.5 M $MgCl_2$
2	Vortex briefly to mix.

Precipitation in 96-Well MicroAmp Trays

Equipment required:

- Variable speed table-top centrifuge with microtiter plate tray, capable of reaching at least 1400 × *g*
- Strip caps or adhesive-backed aluminum foil tape (3M Scotch Tape 425-3)[1]

To precipitate extension products in MicroAmp Trays:

Step	Action
1	Remove the MicroAmp Tray from the thermal cycler. Remove the caps from each tube.
2	Add 74 µL of 70% EtOH/0.5 mM $MgCl_2$ to each tube. (Alternatively, add 20 µL of 2 mM $MgCl_2$ and then 55 µL of 95% ethanol.)
3	Seal the tubes by applying a piece of 3M Scotch Tape 425-3 adhesive-backed aluminum foil tape. Press the foil onto the tubes to prevent any leakage.
4	Invert the tray a few times to mix.
5	Leave the tray at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.

1. Contact 3M in the USA at (800) 364-3577 for your local 3M representative. Use of other tapes may result in leakage or contamination of the sample.

To precipitate extension products in MicroAmp Trays: *(continued)*

Step	Action
6	Place the tray in a table-top centrifuge with tube-tray adaptor and spin it at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: ♦ 1400–2000 × *g*: 45 minutes ♦ 2000–3000 × *g*: 30 minutes **Note** A MicroAmp tube in a MicroAmp Tray can withstand 3000 × *g* for 30 minutes. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
7	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the tray onto a paper towel folded to the size of the tray.
8	Place the inverted tray with the towel into the table-top centrifuge and spin at 700 × *g* for 1 minute.
9	Remove the tray and discard the paper towel. **Note** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.

Precipitation in Microcentrifuge Tubes

Reagents and equipment required:

- 1.5-mL microcentrifuge tubes
- Benchtop microcentrifuge, capable of reaching at least 14000 × *g*
- Vacuum centrifuge

To precipitate extension products in microcentrifuge tubes:

Step	Action
1	Pipet the entire contents of each extension reaction into a 1.5-mL microcentrifuge tube. **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 1 on page 3-37
2	Add 74 µL of 70% EtOH/0.5 mM $MgCl_2$ to each tube. (Alternatively, add 20 µL of 2 mM $MgCl_2$ and then 55 µL of 95% ethanol.)
3	Close the tubes and vortex briefly.
4	Leave the tubes at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.
5	Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 20 minutes at maximum speed. **IMPORTANT** Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.

To precipitate extension products in microcentrifuge tubes: *(continued)*

Step	Action
6	Carefully aspirate the supernatant with a separate pipette tip for each sample and discard. Pellets may or may not be visible. **IMPORTANT** The supernatant must be removed completely, as unincorporated dye terminators are dissolved in it. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.
7	Visually inspect the sample tubes for residual supernatant. If there is any residual supernatant: a. Place the tubes in the microcentrifuge in the same orientation as in step 5 and spin for 10 seconds. b. Aspirate the supernatant carefully as in step 6.
8	Rinse the pellet with 250 µL of 70% ethanol.
9	Vortex briefly.
10	Spin for 5 minutes in a microcentrifuge at maximum speed. Again, carefully aspirate the supernatant and discard.
11	Dry the samples in a vacuum centrifuge for 10–15 minutes or to dryness. (Alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90 °C for 1 minute.)

Shrimp Alkaline Phosphatase Digestion

Note This procedure is for use only with the rhodamine dye terminator and dRhodamine terminator chemistries. It can be used for more efficient removal of unincorporated dye terminators, but adds additional time and expense.

Step	Action
1	At the end of thermal cycling, add 2 µL of SAP (1 U/µL) and 18 µL of 1X SAP buffer to each tube. Seal each tube and incubate at 37 °C for 30 minutes.
2	**For precipitation in 96-Well MicroAmp Trays:**
	a. Add 150 µL of 70% EtOH/0.5 mM $MgCl_2$ to each tube. (Alternatively, add 40 µL of 2 mM $MgCl_2$ and then 110 µL of 95% ethanol.) b. Proceed to step 3 of “Precipitation in 96-Well MicroAmp Trays” on page 3-43.
	For precipitation in microcentrifuge tubes:
	a. Transfer the contents of each tube to a 1.5-mL microcentrifuge tube. b. Add 150 µL of 70% EtOH/0.5 mM $MgCl_2$ to each tube. (Alternatively, add 40 µL of 2 mM $MgCl_2$ and then 110 µL of 95% ethanol.) c. Proceed to step 3 of “Precipitation in Microcentrifuge Tubes” on page 3-44.

Preparing Dye Primer Reaction Products for Electrophoresis

Ethanol Precipitation for Fluorescein/ Rhodamine Dye Primers

Reagents and equipment required for these methods:

- 1.5-mL microcentrifuge tubes
- Benchtop microcentrifuge, capable of reaching at least 14000 × *g*
- Vacuum centrifuge
- 95% Ethanol (ACS reagent grade), non-denatured

Note These procedures do not use salt.

IMPORTANT Use non-denatured 95% ethanol rather than absolute (100%) ethanol. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some protocols.

Method 1

Step	Action
1	Add 80 µL of 95% ethanol to a clean microcentrifuge tube. **Note** The use of sodium acetate is not necessary for precipitation.
2	Pipet the extension reactions from the bottom of each of the four tubes into the ethanol mixture. Mix thoroughly. **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 1 on page 3-37.
3	Place the tube on wet ice or leave it at room temperature for 10–15 minutes to precipitate the extension products.
4	Spin the tube in a microcentrifuge for 10–20 minutes at maximum speed. Carefully aspirate the supernatant and discard. At this point, a pellet may or may not be visible.
5	**Optional:** Rinse the pellet with 250 µL of 70% ethanol and spin for 5 minutes in a microcentrifuge. Again, carefully aspirate the supernatant and discard. This may remove some of the salts from the pellet, but doing so is often not necessary. **Note** If you use sodium acetate, you must rinse the pellet. This will reduce the carryover of salt.
6	Dry the pellet in a vacuum centrifuge for 1–3 minutes, or until dry. Do not overdry.

Method 2

Step	Action
1	Add 80 µL of 95% ethanol to the “A” reaction tube. **Note** This method will not work if the TC1 or DNA Thermal Cycler 480 was used for thermal cycling.
2	Transfer the contents of the “A” reaction tube into the “C” reaction tube.
3	Pipet that mixture into the “G” reaction tube and so on until the contents of all four reaction tubes have been transferred into a single 1.5-mL microcentrifuge tube.
4	Spin the tube in a microcentrifuge for 10–20 minutes at maximum speed. Carefully aspirate the supernatant and discard. At this point, a pellet may or may not be visible.

Method 2 *(continued)*

Step	Action
5	**Optional:** Rinse the pellet with 250 µL of 70% ethanol and spin for 5 minutes in a microcentrifuge. Again, carefully aspirate the supernatant and discard. This may remove some of the salts from the pellet, but doing so is often not necessary. **Note** If you use sodium acetate, you must rinse the pellet. This will reduce the carryover of salt.
6	Dry the pellet in a vacuum centrifuge for 1–3 minutes, or until dry. Do not overdry.

Ethanol Precipitation for BigDye Primers

Reagents and equipment required for this method:

- 1.5-mL microcentrifuge tubes
- Benchtop microcentrifuge, capable of reaching at least 14000 × *g*
- Vacuum centrifuge
- 95% Ethanol (ACS reagent grade), non-denatured

Note This procedure does not use salt.

IMPORTANT Use non-denatured 95% ethanol rather than absolute (100%) ethanol. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some protocols.

Method 1

Step	Action
1	Add 53 µL of 95% ethanol (100 µL if sequencing BAC DNA or other high-sensitivity reactions) to a clean microcentrifuge tube. **Note** The use of sodium acetate is not necessary for precipitation.
2	Pipet the extension reactions from the bottom of each of the four tubes into the ethanol mixture. Mix thoroughly. **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 1 on page 3-37.
3	Place the tube on wet ice or leave it at room temperature for 10–15 minutes to precipitate the extension products.
4	Spin the tube in a microcentrifuge for 10–20 minutes at maximum speed. Carefully aspirate the supernatant and discard. At this point, a pellet may or may not be visible.
5	**Optional:** Rinse the pellet with 250 µL of 70% ethanol and spin for 5 minutes in a microcentrifuge. Again, carefully aspirate the supernatant and discard. This may remove some of the salts from the pellet, but doing so is often not necessary. **Note** If you use sodium acetate, you must rinse the pellet. This will reduce the carryover of salt.
6	Dry the pellet in a vacuum centrifuge for 1–3 minutes, or until dry. Do not overdry.

Method 2

Step	Action
1	Add 53 µL of 95% ethanol (100 µL if sequencing BAC DNA or other high-sensitivity reactions) to the “A” reaction tube. **Note** This method will not work if the TC1 or DNA Thermal Cycler 480 was used for thermal cycling.
2	Transfer the contents of the “A” reaction tube into the “C” reaction tube.
3	Pipet that mixture into the “G” reaction tube and so on until the contents of all four reaction tubes have been transferred into a single 1.5-mL microcentrifuge tube.
4	Spin the tube in a microcentrifuge for 10–20 minutes at maximum speed. Carefully aspirate the supernatant and discard. At this point, a pellet may or may not be visible.
5	**Optional:** Rinse the pellet with 250 µL of 70% ethanol and spin for 5 minutes in a microcentrifuge. Again, carefully aspirate the supernatant and discard. This may remove some of the salts from the pellet, but doing so is often not necessary. **Note** If you use sodium acetate, you must rinse the pellet. This will reduce the carryover of salt.
6	Dry the pellet in a vacuum centrifuge for 1–3 minutes, or until dry. Do not overdry.

Express Load Option for BigDye Primers Run on 36-Lane Gels

Note This procedure is for use only with the BigDye primers.

Reagents and equipment required for this method:

- 0.2-mL PCR tubes
- Benchtop microcentrifuge, capable of reaching at least 14000 × *g*
- Vortexer
- Ethylenediaminetetraacetic acid, disodium salt (Na_2EDTA), 5 mM
- 25 mM EDTA (pH 8.0)with 50 mg/mL blue dextran (P/N 402055)
- Deionized formamide

To perform express load with BigDye primers:

Step	Action
1	Combine the four reactions (A, C, G, T) with 5 µL of 5 mM EDTA (25 µL total volume).
2	Vortex briefly, then spin in a microcentrifuge.
3	Prepare a loading buffer by combining the following in a 5:1 ratio: ♦ deionized formamide ♦ 25 mM EDTA (pH 8.0) with blue dextran (50 mg/mL) **! WARNING ! CHEMICAL HAZARD Formamide is a teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling.**
4	Combine 4 µL of each 25-µL reaction/EDTA mixture with 4 µL of loading buffer in a fresh tube. Store the remaining reaction/EDTA mixture at –15 to –25 °C. This mixture can be concentrated by ethanol precipitation if the Express Load procedure does not yield enough signal.
5	Heat the samples at 98 °C for 5 minutes with the lids open to denature and concentrate the sample. Place on ice until ready to load.
6	Load 2.5 µL of each sample onto the gel.

Preparing and Loading Samples for Gel Electrophoresis

Loading Recommendations

The amount of sample to load depends on many factors, including:

- Sequencing chemistry used
- Quality and nature of the DNA template
- Primer performance
- Instrument configuration (*i.e.*, which instrument and the number of gel lanes)

In general, a very clean template with a perfectly matched primer of good cycle sequencing characteristics will produce enough signal. However, GC-rich templates tend to give weaker signals, so more of the sample should be loaded.

The optimal range of total signal strength is 400–4000. For dRhodamine-based chemistries, the lower limit can be 200. The total signal is calculated by adding the signals for all four bases. These numbers are located in the annotation view of the analyzed sample file (see page 7-8) and at the top of the electropherogram printout.

Acceptable sequence can be obtained from data with total signal strength below these numbers. However, the background will be more noticeable and can interfere with basecalling, especially at the end of the run. If the total signal strength is below 400 (200 for dRhodamine-based chemistries) and the quality of the data is unacceptable, more of the reaction needs to be loaded. If 100% of the reaction was already loaded, try any of the following:

- Check the quantitation and purity of the template. Adjust the amount of template. Sometimes adding 25–50% more template can raise signals.
- Use a high-sensitivity sequencing method (2X volume reaction).
- Repurify the template to ensure that salt and ethanol contaminants are removed.
- Ensure that the primer is suitable for the template and has good melting characteristics (ideally having a T_m of approximately 50–60°C).

If you are running these chemistries for the first time, use the upper limit of the load amount (Table 3-4 through Table 3-6 on page 3-51). Having more signal than you need is better than having an insufficient amount of signal to analyze the data.

Preparing Loading Buffer

Prepare the loading buffer by combining the following in a 5:1 ratio:

- Deionized formamide (see page A-16 for preparation)
- 25 mM EDTA (pH 8.0) with 50 mg/mL blue dextran (P/N 402055)

! WARNING ! CHEMICAL HAZARD. Formamide is a teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling.

Note With ABI™ 373 instruments, you can use 50 mM EDTA (with or without blue dextran) in place of 25 mM EDTA with blue dextran. Do not use 50 mM EDTA on ABI PRISM 377 instruments.

Sample Loading Volumes

ABI 373 and ABI 373 with XL Upgrade

Table 3-4 Loading Amounts for Different Comb Sizes and Chemistries

Chemistry	Volume (µL)	18-well	24-well	36-well	48-well	64-well
Rhodamine Dye Terminator	Resuspend	4–6	4–6	3–4	2	2
	Load	1–2	1–2	3–4 (24, 34 cm) 2–4 (48 cm)	1–2	1–1.5
Fluorescein/ Rhodamine Dye Primer	Resuspend	4–6	4–6	3–4	2	2
	Load	4–6	4–6	3–4 (24, 34 cm) 2–4 (48 cm)	1–2	1–1.5

ABI 373 and ABI 373XL with BigDye Filter Wheel

Table 3-5 Loading Amounts for Different Comb Sizes and Chemistries

Chemistry	Volume (µL)	18-well	24-well	36-well	48-well	64-well
dRhodamine Terminator	Resuspend	4	4	4	2	2
	Load	2–4	2–4	2–4	1–2	1–2
BigDye™ Terminator	Resuspend	4	4	4	4	4
	Load	1–2	1–2	1–2	1–2	1–2
BigDye™ Primer	Resuspend	4	4	4	4	4
	Load	1–2	1–2	1–2	1–2	1–2

ABI PRISM 377 (All Models)

Table 3-6 Loading Amounts for Different Comb Sizes and Chemistries

Chemistry	Volume (µL)		18-well	36-well	48-well	64-well	96-well
Rhodamine Dye Terminator	Resuspend		6	6	4–6	4–6	4–6
	Load		1.5	1.5	1.5	1.5	1.5
dRhodamine Terminator	Resuspend		4	4	2	2	2
	Load		2	2	1	1	1
BigDye Terminator	1X reactions	Resuspend	6–8	6–8	4–6	4–6	4–6
		Load	0.75–1.5	0.75–1.5	0.5–1	0.5–1	0.5–1
	0.5X reactions	Resuspend	2	2	2	2	2
		Load	2	2	1.5–2	1–1.5	1–1.5
	2X reactions	Resuspend	2	2	1.5	1.5	1.5
		Load	2	2	1.5	1.5	1–1.5
Fluorescein/ Rhodamine Dye Primer	Resuspend		6	6	4–6	4–6	4–6
	Load		1.5	1.5	1.5	1.5	1.5
BigDye Primer	1X reactions	Resuspend	6	6	4–6	4–6	4–6
		Load	1–1.5	1–1.5	1	1	1
	0.5X reactions	Resuspend	2–4	2–4	1–2	1–1.5	1–2
		Load	2	2	1	1	1
	2X reactions	Resuspend	2	2	1.5	1	1.5
		Load	2	2	1.5	1	1.5

Loading Samples

Step	Action
1	Resuspend each sample pellet in the appropriate volume of loading buffer according to Table 3-4 through Table 3-6 on page 3-51.
2	Vortex and spin the samples.
3	Heat the samples at 95 °C for 2 minutes to denature, then place on ice.
4	Load the appropriate volume of each sample into a separate lane of the gel according to Table 3-4 through Table 3-6 on page 3-51.

Preparing and Loading Samples for Capillary Electrophoresis

Minimum Sample Volume

The minimum sample volume is 10 µL.

One sample can be injected several times, because very little sample volume is used for each injection.

Preparing the Samples

Step	Action
1	Add 12–25 µL of TSR (see page 5-2) to each sample pellet. **Note** Because injection is electrokinetic, the resuspension volume is not as important as for slab gel electrophoresis.
2	Mix thoroughly on a vortex mixer and heat for 2 minutes at 95 °C. **Note** If you are using a 96-well tray, samples can be denatured directly in the tray.
3	Chill on ice, vortex thoroughly, then spin briefly in a microcentrifuge.
4	Hold on ice until ready to load on the instrument.
5	Transfer the samples to 0.5-mL or 0.2-mL sample tubes and cover with tube septa. **Note** You must use tube septa to prevent evaporation of samples, especially if samples are put in the autosampler more than six hours before analysis.

Preparing a Portion of a Reaction Mixture for Analysis

Occasionally, you may want to prepare only a portion of a sequencing reaction mixture for analysis on the ABI PRISM® 310 Genetic Analyzer and reserve the rest of the sample for analysis later or elsewhere.

Step	Action
1	Add 6 µL of TSR to the dried DNA sequencing reaction.
2	Vortex to dissolve the extension products.
3	Heat for 1 minute at 95 °C to ensure denaturation.
4	Add 2 µL of the sample to 10 µL of TSR in a sample tube.
5	Cover the tube with a septum and vortex well.
6	Heat the mixture for 2 minutes at 95 °C, then place it on ice until ready to use.

Although not recommended on a routine basis, you can keep samples prepared in TSR frozen for several weeks before running on the ABI PRISM 310 Genetic Analyzer with no detectable loss in resolution or base calling.

Loading the Samples

Move the samples into the autosampler as follows:

Step	Action
1	Transfer the denatured samples to a 48- or 96-well tray. **Note** If you are using a 96-well tray, samples can be denatured directly in the tray. **IMPORTANT** The tube arrangement and order of the samples in the tray and on the Sample Sheet must be the same. Make note of the tube arrangement you use, so that you can prepare the Sample Sheet correctly.
2	Seal each tube with a septum, and place the tray into the autosampler.
3	Refer to the ABI PRISM 310 *Genetic Analyzer User's Manual* for electrophoresis procedures.

Optimizing Gel Electrophoresis 4

Introduction

In This Chapter

This chapter describes the following:

- Theory of polyacrylamide gels
- Reagents used to make sequencing gels
- Avoiding problems with sequencing gels

Refer to page 7-44 for information on troubleshooting gel electrophoresis.

Theory of Polyacrylamide Gels

Many variables are involved in determining the number of bases you can expect to read when sequencing DNA. One of the most important of these is the polyacrylamide gel.

Polyacrylamide gels are formed by co-polymerization of acrylamide and bisacrylamide. The reaction is a vinyl addition polymerization initiated by a free radical generating system:

- Polymerization is initiated by TEMED (tetramethylethylenediamine) and APS (ammonium persulfate). The TEMED acts as an electron carrier to activate the acrylamide monomer, providing an unpaired electron to convert the acrylamide monomer to a free radical.
- The activated monomer then reacts with an unactivated monomer to begin the polymer chain reaction.
- The elongating polymer chains are randomly crosslinked by bisacrylamide, resulting in closed loops and a complex web polymer with a reproducible porosity that depends on the polymerization conditions and monomer concentration.

Polymerization depends on factors such as concentration of initiator, temperature, pH, additives, breakdown products and impurities in the chemicals and water used. Light and high temperature can cause autopolymerization of linear acrylamide, which hinders the reproducibility of gel formation.

When used in conjunction with the ABI™ 373 or ABI PRISM® 377 DNA Sequencer, a typical gel made with good reagents and properly polymerized can separate DNA fragments from 1–600 bases in length easily. An exceptional gel used on the ABI 373 or ABI PRISM 377 DNA Sequencer can yield basecalling beyond 900 bases.

For more information, refer to *Acrylamide Polymerization—A Practical Approach* on the Bio-Rad Laboratories World Wide Web site (http://www.bio-rad.com/38973.html).

Reagents

Acrylamide

! WARNING ! CHEMICAL HAZARD. Acrylamide and bisacrylamide are poisons, neurotoxins, irritants, carcinogens, and possible teratogens. Acrylamide and bisacrylamide sublime (the solids release toxic vapor) and are harmful if swallowed, inhaled, or absorbed through the skin. Effects are cumulative. When handling, always wear protective equipment (lab coat, safety glasses, and chemical-resistant gloves) and use in a well-ventilated area. On a routine basis, thoroughly clean surfaces subject to contamination.

Use fresh, high-quality acrylamide. Poor quality acrylamide contains acrylic acid (a deamidation product) and linear polyacrylamide, which will copolymerize and cause local pH changes in the gel. This causes streaking and smearing of bands (Figure 7-50 on page 7-44).

Store acrylamide solutions at 2–6 °C up to 1 month. During storage, especially in water, acrylamide breaks down into acrylic acid. It decomposes more quickly at room temperature.

Urea

! WARNING ! CHEMICAL HAZARD. Urea is a potential mutagen. Dangers cited in toxicity studies show reproductive and tumorigenic effects. Urea can cause irritation to the skin, eyes, and respiratory tract. Avoid inhalation and contact with skin, eyes, and clothing. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.

Urea, $(NH_2)_2CO$, slowly decomposes in water, forming ammonia and cyanate ions that can interfere with electrophoresis. Avoid heating solutions containing urea as much as possible. Cyanate formation is accelerated with increasing temperature. Urea solutions can be pretreated with mixed-bed resins to reduce the amount of ions in solution.

TBE Buffer

Prepare Tris-borate-EDTA buffer with Tris base (see page A-15 for recipe).

If Tris-HCl is used to prepare buffers, the concentration of salt will be too high and nucleic acids will migrate anomalously, yielding extremely diffuse bands.

APS

! WARNING ! CHEMICAL HAZARD. Ammonium persulfate (APS) is harmful if swallowed, inhaled, or absorbed through the skin. It is extremely destructive to mucous membranes, eyes, and skin. Inhalation can be fatal. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.

APS is very hygroscopic and reactive by nature and is prone to reduction and decomposition. It begins to break down almost immediately when dissolved in water.

Optimally, APS should be prepared fresh daily. At the very least, store 10% stocks at –15 to –25 °C and replace every week. Listen for a crackling sound when adding water to dry APS. Material that has broken down due to high humidity or liquid contamination will no longer crackle when moistened.

Persulfate is consumed in the polymerization because it provides the free radicals that drive polymerization, but excess APS can cause oxidation of proteins and nucleic acids. Prerunning gels keeps excess APS traveling ahead of the sample.

TEMED

! WARNING ! CHEMICAL HAZARD. TEMED (Tetramethylethylenediamine) is extremely flammable, and can be very destructive to the skin, eyes, nose, and respiratory system. Keep TEMED in a tightly closed container. Avoid inhalation and contact with the skin, eyes, and clothing. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.

TEMED oxidizes readily, which causes a gradual loss in catalytic activity. It is hygroscopic and accumulates water which accelerates oxidative decomposition. Discard it as chemical waste when oxidation products begin to give it a yellow color. For this reason, use colorless tips to add TEMED.

TEMED introduces fixed charges into the gel matrix that cause gradient drift towards the anode. Unreacted TEMED will focus at the cathode and produce a more alkaline pH gradient. Because TEMED is only active as a free base, polymerization is inhibited at low pH. Use of more than the recommended volume of TEMED will result in brittle gels.

TEMED and APS Concentrations

The properties of the gel depend on the concentrations of APS and TEMED:

- Optimal amounts of APS/TEMED result in long chain lengths, low turbidity, and gel elasticity. These are desirable properties.
  - If concentrations of APS/TEMED are too low, polymerization will be too slow. When polymerization is too slow, oxygen can enter the monomer solution during the process and inhibit polymerization further. Too little polymerization leads to extension products migrating too quickly.
  - If concentrations of APS/TEMED are too high, chain initiation events increase, resulting in shorter chain lengths, higher gel turbidity, and decreased gel elasticity. Low percentage acrylamide gels are most sensitive to excess initiator concentrations.
- Excess TEMED increases buffer pH.
- Excess APS acts as a buffer between pH 8 and 9.
- If you use degraded TEMED or APS, the concentration of initiators will be less than that recommended in the protocol.

Avoiding Problems with Sequencing Gels

Formulation

Use the correct gel formulation for your instrument and application. See Appendix A for gel preparation protocols.

Contaminants

Contaminants cause a variety of problems that affect the quality of sequence data adversely. Contaminants in gels can come from many sources:

- Glassware used to make the gel
- Sinks used to clean gel plates and glassware
- Sponges used to clean gel plates
- Colored paper towels
- Water used for cleaning or gel formulation
- Solvents used for cleaning glass plates
- Marking pens

Background Fluorescence

Background fluorescence on the gel is a problem because it masks the signals from the dyes used to detect sequencing extension products. It can be avoided by careful handling of glass plates, spacers, combs, and buffer chambers.

Do not use any kind of ink on plates, spacers, combs, and buffer chambers. Ink fluoresces strongly, obscuring data (Figure 7-52 on page 7-46).

Washing plates in communal sinks where fluorescent products are disposed can also lead to background fluorescence problems.

Polymerization

The properties of the gel depend on the rate of polymerization. The rate of polymerization is affected by temperature, initiator (APS and TEMED) concentrations, oxygen, and contaminants in the reagents used to formulate gels.

Temperature

Controlling the temperature is crucial for achieving reproducible gels because it directly affects the polymerization time and thus affects the gel properties. A gel formed in a cold environment (such as a room at 2–6 °C) will be turbid, porous, inelastic, and the run-to-run reproducibility will be greatly compromised. Polymerization in a 20–25 °C room is optimal.

In addition to the temperature of the room, it is important that the gel solution and glass plates also be at a temperature of 20–25 °C. Gels formed at this temperature will be transparent, less porous, more elastic, and more reproducible. If the temperature is too high, the polymer chains will be shorter and the resulting gel inelastic.

TEMED and APS Concentrations

See page 4-3 for information about TEMED and APS concentrations.

Oxygen

Oxygen acts as a free radical trap, thereby inhibiting polymerization. The result is a porous gel. To prevent the problems caused by oxygen, the following conditions should be met:

- Polymerization must be fast enough to prevent too much oxygen from dissolving into the gel solution during polymerization.

  As long as you use fresh high-quality reagents and follow a standard protocol, this should not be a problem.

- Minimize the amount of oxygen dissolved in the gel solution prior to casting the gel since it can interfere with the rate of polymerization.

  Partial degassing can be accomplished during the vacuum filter step of gel preparation. It is important to keep the vacuum strength and time constant during this step for run-to-run reproducibility. Since cold solutions have a greater capacity for dissolved oxygen, vacuum filtering (and gel casting) should be done with the solution at room temperature. Also, be careful not to introduce bubbles during the stirring and pouring steps of gel casting.

- Air bubbles trapped while casting the gel should be eliminated as they occur.

  Ideally, gels should be poured carefully and gently so that bubbles never form. Tapping gently on the plates while pouring the gel solution will help prevent bubbles from forming.

Using Fresh Gels

For consistent results, use gels within 2–6 hours after casting. Be sure to wait at least 2 hours after casting the gel to ensure complete polymerization, but not longer than 6 hours, as resolution begins to noticeably deteriorate after this time.

Gels that stand overnight can show significantly slower DNA migration due to the slow hydrolysis of urea to ammonium carbonate. Because the amide groups of the polymer slowly hydrolyze into carboxylate groups, gels that stand more than 48 hours may also show significant loss in resolution beyond 350 bases.

If read length is not important for your application, gels can be stored overnight. Wrap the ends of the gels in plastic wrap to prevent drying.

Red Rain

The ABI PRISM 377 DNA Sequencer sometimes produces a gel image having intense, vertical red streaks. This phenomenon, called “red rain,” is usually found near the end of a run (top of the gel image), but can begin much earlier (Figure 7-55 on page 7-49).

Red rain is caused by gel destruction in the read region of the gel. The damaged gel matrix scatters the laser light, with the effect on red light the greatest. Gel destruction often results from drying out of the gel, and is exacerbated by extreme run conditions, *i.e.*, high voltage, high power, high temperature, and long run times.

Therefore, 36-cm, 2400-scan/hr and 48-cm runs are expected to suffer from red rain more readily than 36-cm, 1200-scan/hr runs. However, red rain has been reported to occur in all three standard ABI PRISM 377 run conditions.

The following can be used to help prevent red rain:

- Wrap the gel plates to prevent the gel from drying out.
- Lower the run temperature from 51 °C to 48 °C.
  - A lower temperature results in a slower run speed. Less data will be collected in the same run time.
  - A lower temperature also means less denaturing power in the gel, which can lead to more compressions.

Gel Plate Quality

Use high-quality gel plates. Plates from vendors other than Applied Biosystems may not have the same quality control. Several problems result from poor quality plates, including warping. Figure 7-58 on page 7-52 shows data from plates that warped after 6 months of use.

When plates become warped, the pathlength of the light changes and the laser no longer focuses correctly on the gel. When this occurs on the ABI 373 DNA Sequencer, laser light is scattered back to the detector, causing the gel image to appear blue and green and obscuring data. On the ABI PRISM 377 DNA Sequencer, there is a filter to keep out most of the scattered laser light, but data quality will still suffer.

Gel Plate Cleaning

Cleaning gel plates properly is very important to prevent plate contamination and obtain good data. Plate contamination can cause the following problems with gels:

- Gel extrusion (see page 4-9)
- Artifacts and background from fluorescent contamination (see Figure 7-52 on page 7-46)
- Poor resolution
- Temporary loss of signal (see page 4-9)

Regular Cleaning

We recommend using a laboratory dishwasher with a hot deionized water (90 °C) rinse for regular cleaning.

- The quality of the water and its temperature, pressure, and volume are critical for effective cleaning.
- Rinse residual gel material before placing plates in the dishwasher.
- Use the longest deionized water rinse cycle initially, followed by a drying cycle. After some experimentation, you may be able to reduce the rinse time.
- The use of detergents is not necessary when a dishwasher is used.

If a dishwasher is not available, then wash plates with a dilute solution of Alconox detergent. Rinse with hot water, then rinse again with deionized water.

The following dishwashers have been found to work well:

Dishwasher	P/N	Supplier
Lancer 1600 dishwasher with facility for drying	Lancer 1600 UP	Lancer USA Inc. 705 West Highway 434 Longwood, Florida 32750 Telephone: 407-332-1855 Lancer UK Ltd. 1 Pembroke Avenue Waterbeach, Cambridge CB5 9QR Telephone: 44-01223-861665 Fax: 44-01223-861990
Sequencing plate rack (50 plate capacity) for Lancer dishwasher	SPR 16	Lancer USA Inc. as listed above
Labconco Undercounter Glassware	15-352-801	Fisher Scientific U.S. Headquarters 585 Alpha Drive Pittsburgh, Pennsylvania 15238 Customer Service: 1-800-766-7000 Fax: 1-800-926-1166 Internet: http://www.fishersci.com

We also recommend the following for preventing contamination of gel plates:

- Clean plates as soon as possible following electrophoresis.
- Once dry, avoid excessive handling of the plates with ungloved hands.

Removing Contaminants

The following procedures are not meant to be used for regular gel plate maintenance, but for decontamination. For regular plate cleaning, we recommend using a dishwasher with a hot, deionized water rinse (see page 4-6).

! WARNING ! Preparation of all solutions should be carried out in a hood using safety glasses, gloves, and other appropriate protective clothing.

To perform an alcoholic KOH wash:

Step	Action
1	Add 30–35 g of potassium hydroxide (KOH) or sodium hydroxide (NaOH) pellets to a plastic bottle. **! WARNING ! Potassium hydroxide is hygroscopic and caustic. It can cause severe burns and blindness if it comes in contact with the skin or eyes. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.**
2	Add 200 mL of absolute ethanol to the bottle. **! WARNING ! CHEMICAL HAZARD. Ethanol is a flammable chemical and is irritating to the skin, eyes, respiratory system. It can cause nerve and liver damage, CNS depression, nausea, vomiting, and headache. Always work in a fume hood. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.**

To perform an alcoholic KOH wash: *(continued)*

Step	Action
3	Mix the solution well. It will take at least 15 minutes for most of the pellets to dissolve. **Note** This recipe is for a saturated solution, so some pellets will remain. Store the solution with the bottle capped tightly. During storage, the color of the solution will turn dark red-brown. The solution can still be used, and is good for 1 year.
4	Place some uncolored absorbent towels or other covering in the hood to catch spills.
5	Place the gel plates on the towels with the inside surfaces facing up. **Note** The plates should be nearly level so that the cleaning solution does not run off onto the bench. Only the inside (gel side) surface of the plates need be cleaned, though the outside surfaces can be cleaned similarly.
6	Pour approximately 15 mL of the cleaning solution onto the center of each plate to be cleaned. Spread the solution over the surface of plate.
7	Allow the solution to remain on the plates for 5 minutes. CAUTION **Longer times can harm the plates.**
8	Rinse the plates thoroughly with distilled, deionized water. Allow plates to dry. **Note** Avoid other cleaning procedures or solutions that may reintroduce contaminants to the plates.

An alcoholic KOH wash can also be used to remove buffer chamber gasket marks from the plates.

Step	Action
1	Perform steps 1–5 above.
2	Pour approximately 15 mL of the cleaning solution onto the area of the plate where the gasket mark is.
3	Allow the solution to remain on the plates for 10 minutes. CAUTION **Longer times can harm the plates.**
4	Repeat steps 2 and 3.
5	Rinse thoroughly with deionized water.
6	Clean plates as usual.

To perform a 3 M HCl wash:

Step	Action
1	Place some uncolored absorbent towels or other covering in the hood to catch spills.
2	Pour 10 mL of concentrated HCl (12 N, 37%) carefully into 30 mL of water and mix thoroughly. ! WARNING ! **Hydrochloric acid (HCl) is a very corrosive liquid. Always work in a fume hood to avoid inhalation. Obtain a copy of the MSDS from the manufacturer. Wear appropriate protective eyewear, clothing, and gloves.**

To perform a 3 M HCl wash: *(continued)*

Step	Action
3	Place the plates on the towels with the inside surfaces facing up. **Note** The plates should be nearly level so that the cleaning solution does not run off onto the bench. Only the inside (gel side) surface of the plates need be cleaned, though the outside surfaces can be cleaned similarly.
4	Pour approximately 15 mL of the cleaning solution in the center of each plate to be cleaned. Spread the solution over the surface of plate.
5	Allow the solution to remain on the plates for 5 minutes. **Note** Longer times will not harm the plates but are unnecessary.
6	Rinse the plates thoroughly with distilled, deionized water. Allow plates to dry. **Note** Avoid other cleaning procedures or solutions that can reintroduce contaminants to the plates.

Gel Extrusion

When voltage is applied on the ABI PRISM 377 DNA Sequencer, the polyacrylamide gel sometimes moves from between the glass gel plates toward the cathode (upper electrode) and into the upper buffer chamber. Up to about five centimeters of gel in a folded sheet can be deposited in the chamber.

This “gel extrusion” usually begins at the start of a run or even during the prerun. It is believed to be caused by a buildup of charge on the surface of the glass plate such that the gel is not bound to the plate after pouring. As the voltage is applied, the gel migrates toward the upper electrode.

The gel image can show a variety of anomalous effects, including catastrophic loss of resolution, lane splitting, extreme band tilt, and band distortion (Figure 7-56 on page 7-50).

Almost all known cases of gel extrusion have been resolved by alcoholic KOH washing (see page 4-7) or acid washing (see page 4-8).

Temporary Loss of Signal

This problem manifests itself as a band of little or no signal across the entire width of the gel image. It usually occurs between 150 and 250 bases (see Figure 7-57 on page 7-51). Temporary loss of signal has been traced to contaminants on the gel plates. These contaminants include surfactants, fatty acids, and long chain polymers.

Rinsing glass plates in a dishwasher with hot deionized water (90 °C) has been found in most cases to remove the contaminants that cause temporary loss of signal (see page 4-6).

In a few cases where a dishwasher did not work well, soaking the plates overnight in a 5% solution of Multiterge detergent (VWR Scientific, P/N 34171-010) eliminated the temporary loss of signal.

Optimizing Capillary Electrophoresis 5

Introduction

In This Chapter

This chapter describes the following:

- Differences between capillary and gel electrophoresis
- Capillary electrophoresis consumables
- Optimizing capillary electrophoresis conditions

Refer to page 7-55 for information on troubleshooting capillary electrophoresis.

Capillary Electrophoresis

The large surface area of a capillary allows heat generated during electrophoresis to be dissipated efficiently, allowing high-voltage electrophoresis. The result is rapid, high-resolution separation of DNA fragments. Applied Biosystems takes advantage of this with the ABI PRISM® 310 Genetic Analyzer, a highly automated capillary electrophoresis instrument.

Advantages of Capillary Electrophoresis

Capillary and slab gel electrophoresis both separate DNA fragments by size through a sieving matrix, but there are important differences between the two techniques:

- There is no gel pouring. The ABI PRISM 310 Genetic Analyzer uses a liquid polymer that is pumped automatically into the capillary.
- There is no manual sample loading. The instrument uses electrokinetic injection.
- Run times are shorter.
- Quantitation is more accurate.
- Very little sample is injected, which allows the sample to be run several times if necessary.

Refer to the ABI PRISM 310 *Genetic Analyzer User's Manual* (P/N 903565) for more information.

Capillary Electrophoresis Consumables

Polymer

The polymer is the medium used to separate DNA fragments. There are two types of polymer available for DNA sequencing on the ABI PRISM 310 Genetic Analyzer:

- DNA Sequencing Polymer (DSP)
- Performance Optimized Polymer 6 (POP-6™)

POP-6 provides superior performance over DSP because it can be run at a higher temperature and yields greater read lengths.

Note We do not recommend using the POP-6 polymer with fluorescein/rhodamine dye primer chemistry.

IMPORTANT Do not leave polymer on the instrument more than 5 days.

Introduction of any kind of dust into the polymer can cause spikes in the data. We recommended that you minimize any actions that could introduce particles into the polymer:

- Do not leave the POP-6 polymer exposed to the air by leaving the vial with the lid open.
- Do not clean the syringe and gel block with unfiltered water.
- Do not install a capillary that has been sitting on a bench exposed to dust.

Genetic Analyzer Buffer

Genetic Analyzer Buffer is used for electrophoresis. It is supplied in 10X concentration and should be diluted to 1X concentration for use.

- Use 10X Genetic Analyzer Buffer with EDTA (P/N 402824) with uncoated capillaries. If you do not use buffer with EDTA, capillary life will be shortened greatly.
- Change the buffer every 2–3 days.

Note Use 10X Genetic Analyzer Buffer without EDTA with coated capillaries, which are used only with DNA Sequencing Polymer.

TSR

The cycle sequencing protocols on the ABI PRISM 310 Genetic Analyzer use a sample preparation reagent called Template Suppression Reagent (TSR). TSR is used to prevent high molecular weight species from being injected into and clogging the capillary.

- Store TSR at 2–6 °C.
- At room temperature, samples in TSR are stable for a maximum of 48 hours.
- Although not recommended on a routine basis, you can keep samples prepared in TSR frozen for several weeks before running on the ABI PRISM 310 Genetic Analyzer with no detectable loss in resolution or base calling.

Capillaries

The capillary has an opaque, polyimide external coating except in the window area. The laser and detector read samples during electrophoresis through the window in the coating. Capillaries are very fragile in the uncoated window area.

Capillaries should last at least 100 runs if treated properly. You may be able to get more injections from a capillary, depending on your template preparation methods and run conditions.

- Do not let capillaries with polymer in them dry out. Store their ends in buffer or deionized water when not in use.
- Store unused capillaries in a dust-free environment.
- Do not touch capillary windows. If you do touch a window accidentally, clean it with 95% ethanol.

Signs of capillary failure are the following:

- Decreased resolution
- High baseline
- Noisy data
- Trailing peaks in data (Figure 7-59 on page 7-55)

Information about the capillaries used on the ABI PRISM 310 Genetic Analyzer is given in Table 5-1.

Table 5-1 Capillary Types

Capillary Type	Marking Color	Polymer Used	Coated/ Uncoated	10X Genetic Analyzer Buffer Type	Length (cm)	Length to Detector (cm)	Internal Diameter (µm)
Rapid sequencing	green	POP-6™	internally uncoated	with EDTA	47	36	50
Long-read sequencing	pink	POP-6	internally uncoated	with EDTA	61	50	50
Sequencing	silver	DNA Sequencing Polymer (DSP)	internally coated	without EDTA	47	36	75

Optimizing Electrokinetic Injection

Introduction

Optimizing electrokinetic injection can greatly improve data quality and run-to-run reproducibility. The goal is to inject sufficient DNA to yield peaks of adequate height (that is, data with a good signal-to-noise ratio) while maintaining resolution and read length.

The ABI PRISM 310 run modules have preset values for injection times and voltages. These values are adequate for most applications. However, you should consider modifying the injection parameters if the signal is too strong or too weak or if the resolution is poor.

Signal Too Strong

- Decrease the injection time.
- Decrease the injection voltage.

Signal Too Weak

- Increase the injection time.

  The default injection time is 30 seconds. We do not recommend injection times >120 seconds.
- Increase the injection voltage.

  Increase the voltage in 1–2-kV increments. The maximum possible injection voltage is 15 kV.
- Reduce the amount of salt in the sample.

IMPORTANT Negative ions, *e.g.*, EDTA and acetate, compete with DNA for injection. To reduce the amount of salt in a sequencing reaction, use a spin column (see page 3-34).

Poor Resolution

- Decrease the injection time.

  This will decrease the signal strength, so you may need to increase the injection voltage.

Modifying Injection Time

When you modify the injection time, you will encounter a tradeoff between signal strength and resolution.

- Signal strength (as measured both by peak height and by peak area) increases linearly with increasing injection time for most applications (Figure 5-1 and Figure 5-2 on page 5-5).
- However, an n-fold increase in injection time does not result in an n-fold increase in peak height. In Figure 5-1, no improvement is seen after 10 seconds for the larger fragment. The signal decreases dramatically after 40 seconds for the smaller fragment.
- As the injection time increases, the resolution decreases because of increasing peak widths (Figure 5-3 on page 5-5). There is too much sample to move as a discrete, well-resolved band.

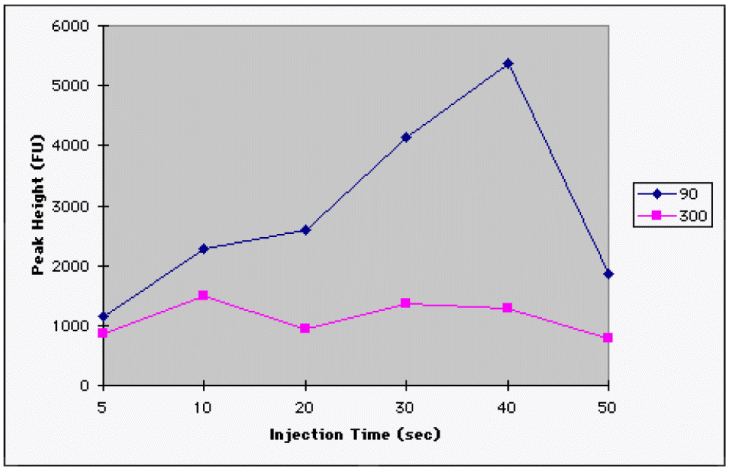


Figure 5-1 Peak height vs. injection time for two different-sized fragments (90 bp and 300 bp)

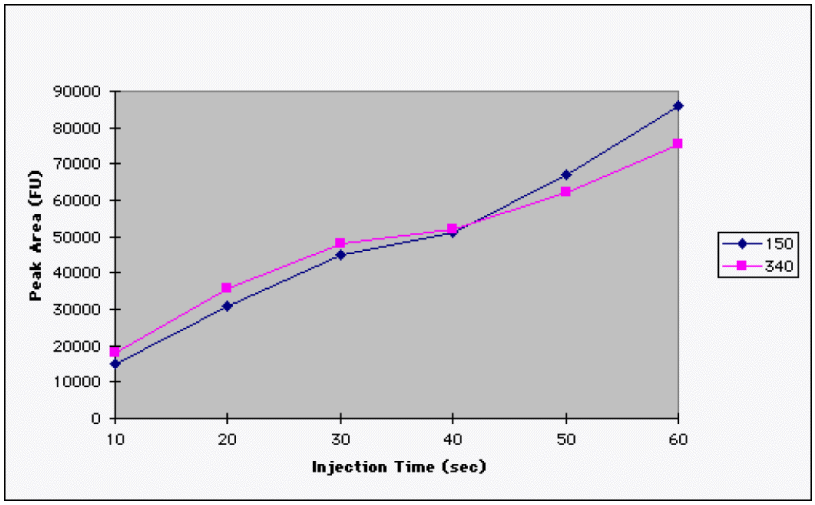


Figure 5-2 Peak area vs. injection time for two different-sized fragments (150 bp and 340 bp)

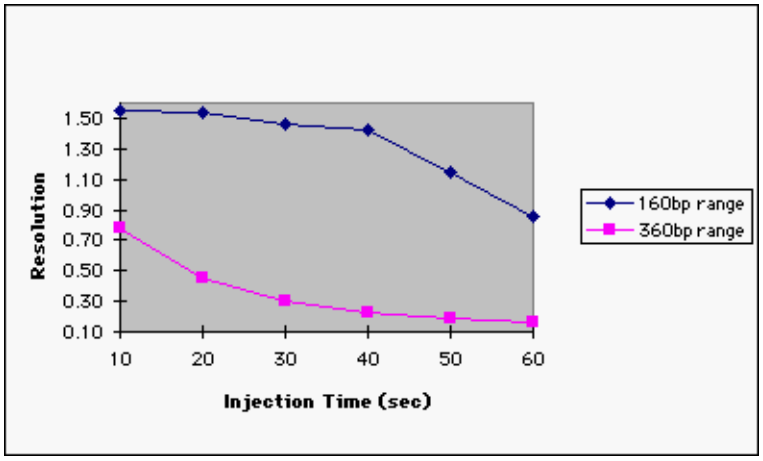


Figure 5-3 Resolution vs. injection time for different-sized fragments

Modifying Injection Voltage

Injection voltage has little effect on peak resolution. Resolution with injection voltages of 319 V/cm (the highest possible setting) is often indistinguishable from resolution with injection voltages of 53 V/cm (a typical value for a 47-cm capillary).

Peak height and peak area increase linearly with increasing injection voltage.

Setting Electrokinetic Injection Values

For information on setting electrokinetic injection values, refer to the ABI PRISM 310 *Genetic Analyzer User's Manual*.

Optimizing Electrophoresis Conditions

Introduction

Optimizing electrophoresis conditions (run time, run voltage, and run temperature) can greatly improve data quality, run-to-run reproducibility, and/or throughput. When selecting values for these parameters, consider the following factors:

- Read length desired
- Required degree of resolution

Run Time

Determining Required Run Time

To ensure that you collect sufficient data to perform analysis, set the electrophoresis run time approximately 10% higher than the migration time of the longest fragment you want to detect.

- For sequencing samples using DSP, the standard run voltage is 160 volts/cm. For a 47-cm capillary, this translates to 7.5 kV. The current at this voltage is 7–10 µA. The time required for a 450-base fragment to reach the detector window is about 135 minutes with these run conditions.
- For sequencing samples using POP-6 and long-read sequencing, the standard run voltage is 200 volts/cm. For a 61-cm capillary, this translates to 12.2 kV. The current at this voltage is 4–6 µA. The time required for a 600-base fragment to reach the detector window is about 120 minutes with these run conditions.
- For sequencing samples using POP-6 and rapid sequencing, the standard run voltage is 320 volts/cm. For a 47-cm capillary, this translates to 15 kV. The current at this voltage is 5–8 µA. The time required for a 400-base fragment to reach the detector window is about 35 minutes with these run conditions.

Changing Run Time

You can change the data collection time for special requirements. For example, you can shorten the data collection time if you only need information about short extension products, *e.g.*, in PCR sequencing.

Run Temperature

Protocols for sequencing applications with POP-6 specify a 50 °C electrophoresis temperature.

For templates that do not denature readily, the run temperature can be increased by 1–2 °C, however, there is a tradeoff between run temperature and resolution.

Laboratory Temperature and Humidity

The laboratory temperature should be maintained between 15 and 30 °C. It should not fluctuate more than ±6 °C during a run for optimal results.

The ABI PRISM 310 Genetic Analyzer can tolerate up to 80% non-condensing relative humidity. Avoid placing the instrument near heaters, cooling ducts, or windows.

For More Information

For information on setting electrophoresis parameters, refer to the ABI PRISM 310 *Genetic Analyzer User's Manual*.

Run Parameters for Specific Sequencing Chemistries

Table 5-2 Reference Table for Specific Sequencing Chemistries

Chemistry	Syringe	Polymer	Run Module	Dye Set/Primer File	Base Caller	Capillary Size	Capillary Mark
Rhodamine Dye Terminator	250 µL	DSP	Seq Run (250 uL) A	DT5%CEHV{A Set–any primer}	CE-1	47 cm x 75 µm	silver
Rhodamine Dye Terminator, long-read sequencing	1.0 mL	POP-6™	Seq POP6 (1.0 mL) A	DT POP 6	CE-2	61 cm x 50 µm	pink
Rhodamine Dye Terminator, rapid sequencing	1.0 mL	POP-6	Seq POP6 (1.0 mL) Rapid A	DT POP 6	CE-2	47 cm x 50 µm	green
dRhodamine Terminator	250 µL	DSP	Seq Run (250 uL) E	DT5%CEHV{dR Set–any primer}	CE-1	47 cm x 75 µm	silver
dRhodamine Terminator, long-read sequencing	1.0 mL	POP-6	Seq POP6 (1.0 mL) E	DT POP6 {dR Set–any primer}	CE-1	61 cm x 50 µm	pink
dRhodamine Terminator, rapid sequencing	1.0 mL	POP-6	Seq POP6 (1.0 mL) Rapid E	DT POP6 {dR Set–any primer}	CE-1	47 cm x 50 µm	green
BigDye™ Terminator, long-read sequencing	1.0 mL	POP-6[a]	Seq POP6 (1.0 mL) E	DT POP6 {BD Set–any primer}	CE-1	61 cm x 50 µm	pink
BigDye Terminator, rapid sequencing	1.0 mL	POP-6	Seq POP6 (1.0 mL) Rapid E	DT POP6 {BD Set–any primer}	CE-1	47 cm x 50 µm	green
Fluorescein/ Rhodamine Dye Primer	250 µL	DSP	Seq Run (250 uL) A	DP5%CEHV{-21M13} or DP5%CEHV{M13rev}	CE-1	47 cm x 75 µm	silver
BigDye™ Primer, long-read sequencing	1.0 mL	POP-6[a]	Seq POP6 (1.0 mL) E	DP POP6 {BD Set-21M13} or DP POP6 {BD Set-M13rev}	CE-1	61 cm x 50 µm	pink
BigDye Primer, rapid sequencing	1.0 mL	POP-6	Seq POP6 (1.0 mL) Rapid E	DP POP6 {BD Set-21M13} or DP POP6 {BD Set-M13rev}	CE-1	47 cm x 50 µm	green

a. The BigDye terminator and BigDye primer chemistries are not used with DNA Sequencing Polymer (DSP) on the ABI PRISM 310 instrument.

Optimizing Software Settings

6

Introduction

In This Chapter This chapter describes the following:

Topic	See page
Choosing a Run Module	6-2
Choosing a Dye Set/Primer (Mobility) File	6-3
Choosing the Correct Basecaller	6-6
Creating an Instrument (Matrix) File	6-7
Setting the Data Analysis Range	6-15

Choosing a Run Module

Overview

A run module file contains all the parameters required for a particular function or application (see page 1-12). On the ABI PRISM® 310, ABI™ 373 with XL Upgrade, and ABI PRISM® 377 instruments, choosing a run module automatically chooses the filter set used to collect the data. If an incorrect run module is chosen for a particular chemistry, the data will be poor, with low resolution and miscalled bases (Figure 7-62 on page 7-62). If this happens, rerun the samples using the correct run module.

On ABI 373 instruments there are no run modules, so the filter set and electrophoresis parameters must be chosen manually. For fluorescein/rhodamine dye primer and rhodamine dye terminator chemistries, Filter Set A is used. For dRhodamine-based chemistries on instruments with the BigDye™ Filter Wheel, Filter Set A is also used, but the filters have different wavelengths (see page 1-8).

List of Run Modules

Table 6-1 Run Modules

Instrument	Filter Set A	Filter Set E
ABI PRISM 310	Seq Fill Capillary Seq POP6 (1 mL) A Seq POP6 Rapid (1 mL) A Seq Run (250 uL) A Test CCD 4-Color	Seq Fill Capillary Seq POP6 (1 mL) E Seq POP6 (250 uL) E Seq POP6 Rapid (1 mL) E Seq Run (250 uL) E Test CCD 4-Color
ABI 373 with XL Upgrade	Plate Check Pre Run Seq Run	
ABI PRISM 377 (All Models)[a]	Plate Check Plate Check A Seq PR 36A-1200 Seq PR 36A-2400 Seq Run 36A-1200 Seq Run 36A-2400 Seq Run 48A-1200	Plate Check Plate Check A[b] Plate Check E[c] Seq PR 36A-1200[b] Seq PR 36A-2400[b] Seq PR 36E-1200[c] Seq PR 36E-2400[c] Seq Run 36E-1200 Seq Run 36E-2400 Seq Run 48E-1200

a. Older versions of the ABI PRISM 377 Collection Software may use different nomenclature for run modules, *e.g.*, PR 2X A = Seq PR 36A-1200, Run 4X A = Seq Run 36A-2400.

b. Any plate check and prerun module can be used on the ABI PRISM 377 DNA Sequencer.

c. For the ABI PRISM 377 DNA Sequencer with 96-Lane Upgrade only.

Choosing a Dye Set/Primer (Mobility) File

Overview

The different dyes affect the electrophoretic mobility of cycle sequencing extension products to varying degrees, especially on shorter fragments. However, these mobility shifts are consistent for each dye set and can be corrected readily during analysis.

The analysis software is able to compensate for these mobility differences by applying mobility shifts to the data so that evenly spaced peaks are presented in the analyzed data. The files that contain the mobility shift information are called dye set/primer (mobility) files.

The dye set/primer files available are listed on page 6-5. The files are located in the ABI folder within the System folder on the Macintosh® or Power Macintosh® computer. They are selected in the sample sheet during instrument setup. They can also be selected in the Sequencing Analysis software if samples need to be reanalyzed.

Using the Wrong Dye Set/Primer File

The seriousness of choosing the wrong dye set/primer file (mobility) depends on which mobility file you choose to analyze the data:

- Analyzing fluorescein/rhodamine dye primer data with the wrong fluorescein/rhodamine dye primer mobility file causes shifted peaks.
- Analyzing fluorescein/rhodamine dye primer data with rhodamine dye terminator mobility files or vice versa causes both shifted peaks and miscalled bases, because the dyes used for the fluorescein/rhodamine dye primer and rhodamine dye terminator chemistries are different.
- Analyzing fluorescein/rhodamine dye primer data with BigDye primer mobility files causes shifted peaks.
- Analyzing BigDye™ primer data with a mobility file for dRhodamine terminator or BigDye™ terminator chemistry (or vice versa) causes both shifted peaks and miscalled bases (Figure 7-63 on page 7-62). These three chemistries use the same dyes for fluorescence emission, but on different bases from each other. See Chapter 2, especially page 2-14, for more information.
- Analyzing dye terminator chemistry data with the wrong type of dye terminator mobility file can cause both shifted peaks and miscalled bases. The dRhodamine terminators and BigDye terminators use different dyes for different bases, *i.e.*, the dyes for the C and T bases are switched (see page 2-14).

The dye set/primer file used for data analysis is shown in the annotation view of the sample file (see page 7-8) and is also part of the header information on the sequence electropherogram printout. If you do analyze with incorrect information, the data can be reanalyzed with the correct dye set/primer file as described in your user's manual.

Chemistry-Specific Mobility Information

Fluorescein/Rhodamine Dye Primers

The four fluorescent dyes used for fluorescein/rhodamine dye primer sequencing are of two structural types: 5-FAM and JOE molecules are fluorescein dyes, and TAMRA and ROX are rhodamine dyes (see Figure 2-5 on page 2-8). Rhodamine dyes migrate more slowly during electrophoresis than fluorescein dyes.

The dyes affect the mobilities of different primers in ways specific to each primer. The analysis software contains mobility files for the following sequencing primers:

- –21 M13 Forward
- M13 Reverse
- SP6
- T3
- T7
- PI(+)[a]
- PI(–)[a]

a. Used with the Primer Island Transposition Kit.

IMPORTANT For correct data analysis, the dye set/primer file must be specified in the sample sheet before data collection and automatic analysis. If you choose the wrong dye set/primer file, the data can be reanalyzed with a different file after automatic analysis.

The fluorescent dyes appear to interact with the first five bases on the 5´ end of the oligonucleotide to which they are attached. This interaction is responsible for their effect on primer mobility.

If you use custom dye-labeled primers, synthesize them so that their 5´ ends contain the same five bases as the 5´ end of the M13 Reverse primer, CAGGA. Analyze the sequence data with M13 Reverse dye set/primer files. This works well in most cases, as long as the primers are made with the 5-FAM, JOE, TAMRA, and ROX dyes. Contact the Applied Biosystems Custom Oligonucleotide Synthesis Service (see page 3-19 for contact information).

BigDye Primers

–21 M13 Forward and M13 Reverse primers use the same dye set/primer file on the ABI PRISM 377 DNA Sequencer. This is possible because the mobility shifts are very similar for each primer.

Rhodamine Dye Terminators

Rhodamine dye terminator chemistry uses a set of four rhodamine dyes (see Figure 2-1 on page 2-2). Rhodamine dye-labeled DNA fragments migrate true to molecular weight and no mobility correction is needed. However, the dye set/primer file is needed to tell the software which matrix file is used for analysis and which color is associated with each of the four bases.

dRhodamine Terminators and BigDye Terminators

dRhodamine- and BigDye-labeled DNA fragments do not necessarily migrate true to molecular weight. Some mobility correction is required.

Mobility shifts and dye set/primer file names for the dRhodamine Terminators are similar to those for the BigDye Terminators. If a mobility file for the wrong sequencing chemistry is used, C and T bases will be miscalled because of differences in which terminators are labeled with which dyes (see page 2-14).

List of Dye Set/Primer Files

Table 6-2 Dye Set/Primer (Mobility) Files

Instrument	Sequencing Chemistry				
	Rhodamine Dye Terminator	**dRhodamine Terminator**	**BigDye Terminator**	**Fluorescein/ Rhodamine Dye Primer**	**BigDye Primer**
ABI PRISM 310	DT POP6 DT5%CEHV{A Set-AnyPrimer}	DT POP6{dR Set-Any Primer} DT DSP{dR Set-AnyPrimer}	DT POP6{BD Set-Any Primer}	DP5%CEHV{-21M13} DP5%CEHV{M13Rev} DP5%CEHV{SP6} DP5%CEHV{T3} DP5%CEHV{T7}	DP POP6{BD Set-21M13} DP POP6{BD Set-M13 Reverse}
ABI 373 and ABI 373 with XL Upgrade	DT4%Ac{A Set-AnyPrimer} DT6%Ac{A Set-AnyPrimer}			DP4%Acv2{M13Rev} DP4%Ac{-21M13} DP4%Ac{SP6} DP4%Ac{T3} DP4%Ac{T7} DP6%Ac{-21M13} DP6%Ac{M13Rev} DP6%Ac{SP6} DP6%Ac{T3} DP6%Ac{T7} PI+.mob PI-.mob	
ABI 373 and ABI 373XL with BigDye Filter Wheel		373 dRDT	373 BDT		373 BDP(-21) 373 BDP rev
ABI PRISM 377 (All Models)	DT4%Ac{A Set-AnyPrimer}	DT {dR Set Any-Primer}	DT {BD Set Any-Primer}	DP4%Acv2{M13Rev} DP4%Ac{-21M13} DP4%Ac{SP6} DP4%Ac{T3} DP4%Ac{T7} PI+.mob PI-.mob	DP5%LR{BD M13 FWD & REV}

Refer to your instrument user's manual and the *ABI PRISM DNA Sequencing Analysis Software User's Manual* for more specific information on choosing a dye set/primer file.

Choosing the Correct Basecaller

Choosing a Basecaller

The basecaller is the program that determines the individual base identities in a sequence. The Applied Biosystems basecallers differ from each other primarily in the shape of the internal spacing curves.Choosing the most effective basecaller for any given sample file depends on the quality of the data, the type of run, and the run and gel conditions.

Table 6-3 shows basecaller for each run type. You can try other basecallers with your data to see which works best.

Table 6-3 Choosing the Correct Basecaller

If the samples are from a...	Then use...
ABI PRISM 310, rhodamine dye terminator chemistry with POP-6	CE-2
ABI PRISM 310, all other applications	CE-1
24- or 34-cm well-to-read gel on the ABI 373	ABI50
BaseSprinter or 373-18 run on the ABI 373	ABI100
Typical 1200-scan/hr, 36-cm well-to-read gel on the ABI PRISM 377	ABI100
Typical 2400-scan/hr, 36-cm well-to-read gel on the ABI PRISM 377	ABI200
48-cm well-to-read gel on the ABI 373	ABI50
48-cm well-to-read, 5.25% PAGE-PLUS gel on the ABI PRISM 377	ABI50
48-cm well-to-read, Long Ranger or 19:1 or 29:1 polyacrylamide gel on the ABI PRISM 377	ABI100
Run with many insertions or deletions near the end of the run	SemiAdaptive
If the spacing is a negative number	SemiAdaptive
If the spacing is still a negative number with SemiAdaptive	Adaptive
If you noticed problems with run conditions	Adaptive

IMPORTANT Although each basecaller is optimized for a specific type of run, depending on your run conditions you might get better data using a different basecaller. Analyze your data with different basecallers to determine which one works best for your run conditions.

IMPORTANT If you reanalyze a sample file, the previous analysis results are overwritten by the new results. To avoid erasing the previous analysis results, save a copy of the sample file under a different name before you reanalyze.

For more information, refer to the *ABI PRISM DNA Sequencing Analysis Software User's Manual*.

Creating an Instrument (Matrix) File

Overview

You must use an appropriate instrument (matrix) file to get good data. The instrument file contains the information that allows the Sequencing Analysis software to compensate for the spectral overlap between the dyes when data is analyzed. In some cases, using an incorrect instrument file causes analysis to fail. Instrument files vary between different instruments, and between filter sets on a single instrument. An instrument file must be made for each filter set on each instrument.

The appropriate matrix file can be applied to data on subsequent capillary runs or gels on the same instrument, as long as the same filter set is used. This is because the spectral overlap between the four dyes is very reproducible.

When an Instrument File Needs to be Remade

You will need to make a new instrument file if:

- Different dye set used
- Aging filter wheel on an ABI 373 DNA Sequencer
- Changes to any optics occur, *e.g.*, new filter wheel (on an ABI 373 DNA Sequencer) or CCD camera (on an ABI PRISM 310 or ABI PRISM 377 instrument)

If you use the wrong instrument file (Figure 7-65 on page 7-63), you will need to reanalyze the data with the correct instrument file. However, if you collect data using the wrong filter set, you should rerun the samples using the correct filter set.

Refer to the *ABI PRISM DNA Sequencing Analysis Software User's Manual* for instructions on creating a new instrument file or follow the instructions on page 6-8 for creating an instrument file for dRhodamine-based chemistries.

Data Utility Software

The Data Utility software is used to make instrument files. You must put the correct data file for each matrix standard into the correct "box" in the Data Utility application. Table 6-4 shows the correct placement for making a Filter Set E instrument file (Filter Set A on the ABI 373 DNA Sequencer with BigDye Filter Wheel).

Table 6-4 Placement of Standards for dRhodamine-Based Chemistries

Box	Dye Primer Matrix	Taq Terminator Matrix	T7 Terminator Matrix
C…	dR110	dROX	dR6G
A…	dR6G	dR6G	dTAMRA
G…	dTAMRA	dR110	dROX
T…	dROX	dTAMRA	dR110

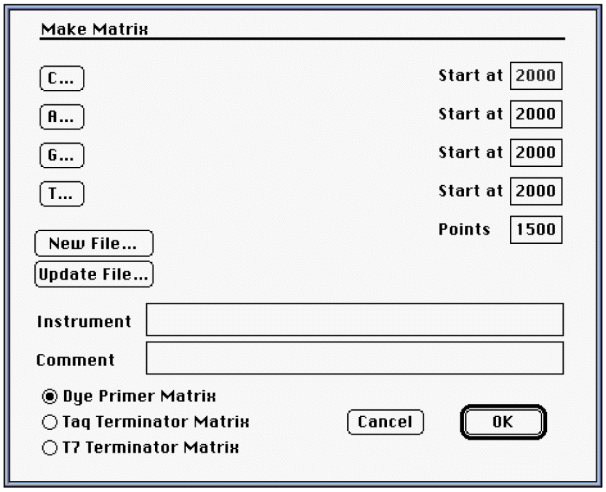
When creating a Filter Set E instrument file, you need to make all three matrix files, even if you are only using one dRhodamine-based chemistry. The data collection software will not run with only a Taq or T7 terminator matrix in the file.

The T7 Terminator Matrix file is needed to analyze dRhodamine terminator and BigDye terminator sequencing data. It has a baselining algorithm associated with it that works well with these chemistries. The dRhodamine terminator and BigDye terminator dye set/primer files have tags in them that tell the Sequencing Analysis software to select this matrix file.

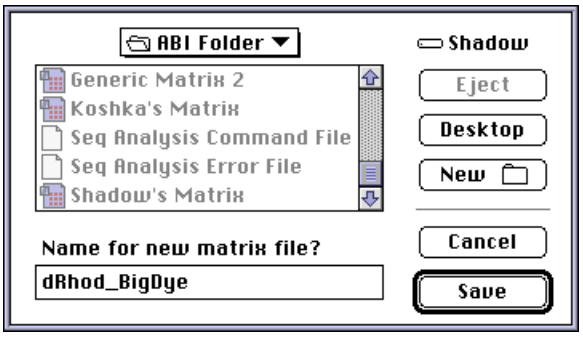
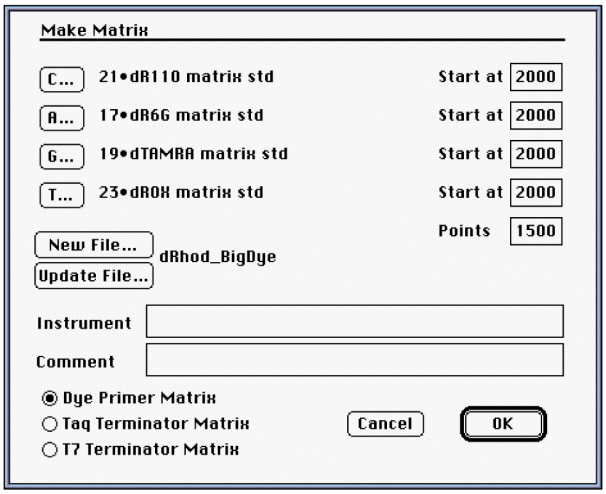
Making a Filter Set E Instrument File from Matrix Standards

This procedure is for making instrument files for dRhodamine-based chemistries (Filter Set E on the ABI PRISM 310 and ABI PRISM 377 instruments, Filter Set A on the ABI 373 DNA Sequencer with BigDye Filter Wheel). Refer to your instrument user's manual for general instructions on creating instrument files.

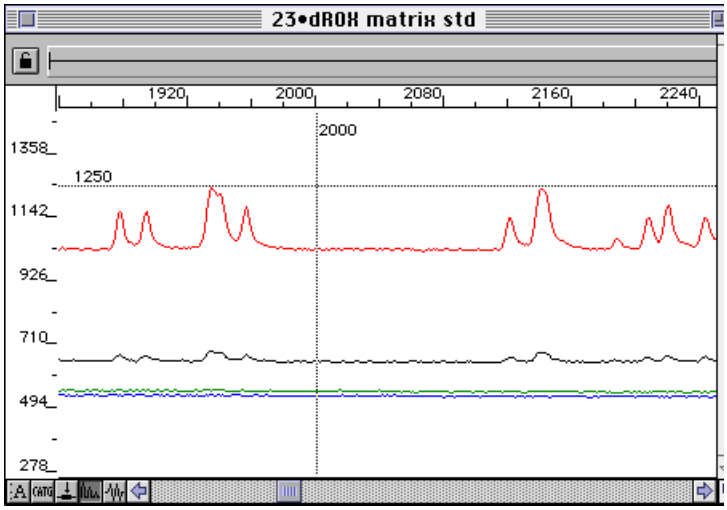
To make the Dye Primer Matrix:

Step	Action
1	Launch the Data Utility software (located in the Utilities folder within the Sequencing Analysis folder).
2	From the Utilities menu, choose Make Matrix… The Make Matrix dialog box appears as shown below. Verify that the Dye Primer Matrix button at the lower left is selected. 
3	Click on the box for each nucleotide base and select the sample file that corresponds to the correct matrix standard as shown in the table below. **Box** / **Dye Primer Matrix** C… / dR110 A… / dR6G G… / dTAMRA T… / dROX
4	For each matrix standard sample, start with the default value of 2000 for the start point. Start with the default value of 1500 for the number of data points to analyze. **Note** If the default values do not work, follow the instructions for using other values in steps 8 and 9 below.

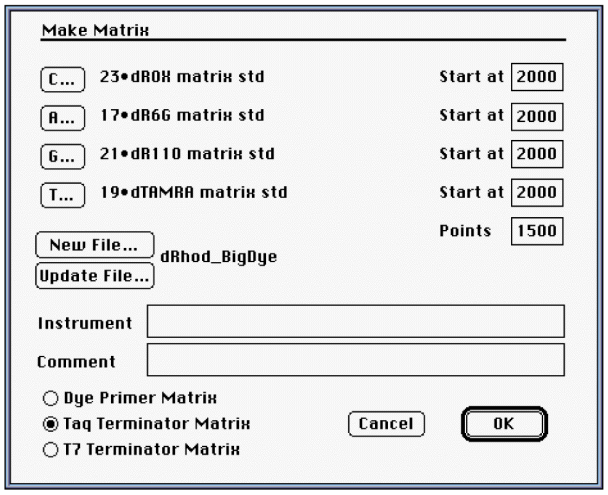
To make the Dye Primer Matrix: *(continued)*

Step	Action
5	Click New File… A dialog window appears as shown below. Name the file dRhod_BigDye (or another appropriate name) and save it in the ABI folder within the System folder. 
6	The Make Matrix dialog box should look like that shown below.  a. Click OK. The computer makes the matrix. When finished, a dialog window appears with the message “Make matrix successfully completed.” b. Click OK.
7	If the computer is unable to make a matrix, examine the raw data again in the Sequencing Analysis software. If you used the default values, then select new start points as directed in steps 8 and 9 below. If many peaks are off-scale, dilute the matrix standards and rerun them.

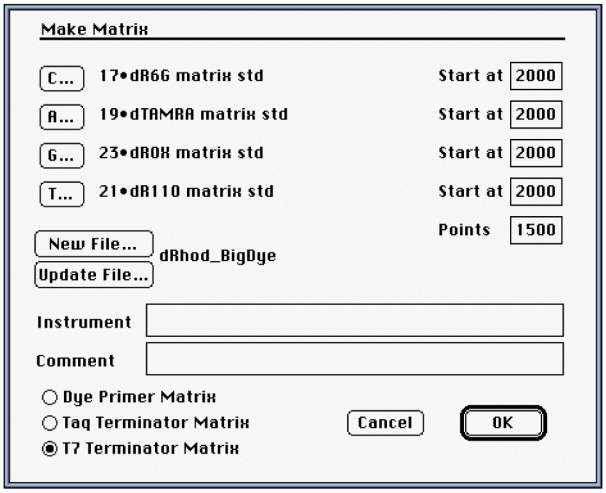
To make the Dye Primer Matrix: *(continued)*

Step	Action
8	If the matrix cannot be made with the default values, proceed with steps a, b, and c below. a. In the Sequencing Analysis software, open a matrix standard sample and examine the raw data. An example is shown below. b. Select a starting point where there are no peaks and the baseline is flat. c. Select a number of data points to analyze such that no peaks in the range are off-scale, *i.e.*, above 4000 relative fluorescence units (RFU), and that the baseline at the end of the range is flat. A typical number of data points is 1500. 
9	Repeat step 8 for each matrix standard sample. Record the results for later use. **IMPORTANT** The number of data points analyzed is the same for each matrix standard. Choose starting points for each sample such that all peaks are less than 4000 RFU and that both the starting and ending points have flat baselines and no peaks.

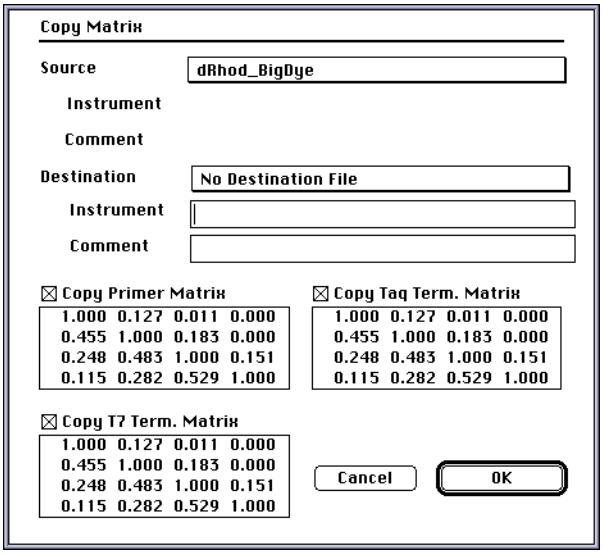
To make the Taq Terminator Matrix:

Step	Action
1	In the Data Utility application, choose Make Matrix… from the Utilities menu. The Make Matrix dialog box appears.
2	In the Make Matrix dialog box, click the Taq Terminator Matrix button at the lower left.
3	Click on the box for each nucleotide base and enter the data file that corresponds to the correct matrix standard as shown in the table below. **Box** / **Taq Terminator Matrix** C… / dROX A… / dR6G G… / dR110 T… / dTAMRA **IMPORTANT** The order of matrix standard data files is different from that in the Dye Primer Matrix (see Table 6-4 on page 6-7).
4	Enter the same numbers for each matrix standard sample in the Start at and Points boxes as were used for the Dye Primer Matrix.
5	Click Update File… A dialog window appears.
6	Choose dRhod_BigDye from the ABI folder within the System folder and click Open. The Make Matrix dialog box should look like that shown below. **Note** The numbers in the Start at and Points boxes are default values. Your numbers may vary. 
7	a. Click OK. The computer makes the matrix. When finished, a dialog window appears with the message “Make matrix successfully completed.” b. Click OK.

To make the T7 Terminator Matrix:

Step	Action
1	In the Data Utility application, choose Make Matrix… from the Utilities menu. The Make Matrix dialog box appears.
2	In the Make Matrix dialog box, click the T7 Terminator Matrix button at the lower left.
3	Click on the box for each nucleotide base and enter the data file that corresponds to the correct matrix standard as shown in the table below (note the order of the matrix standard files). **Box** / **T7 Terminator Matrix** C… / dR6G A… / dTAMRA G… / dROX T… / dR110
4	Enter the same numbers for each matrix standard sample in the Start at and Points boxes as were used in the Dye Primer Matrix and Taq Terminator Matrix.
5	Click Update File… A dialog window appears.
6	Choose dRhod_BigDye from the ABI folder within the System folder and click Open. The Make Matrix dialog box should look like that shown below. **Note** The numbers in the Start at and Points boxes are default values. Your numbers may vary. 
7	a. Click OK. The computer makes the matrix. When finished, a dialog window appears with the message “Make matrix successfully completed.” b. Click OK.

To check the instrument file:

Step	Action
1	From the Utilities menu, choose Copy Matrix…
2	Under Source, select Instrument file and choose dRhod_BigDye from the ABI folder within the System folder. The three matrix files within the dRhod_BigDye instrument file appear as shown below. 
3	Make sure that all three matrix files have numbers that range from 0–1. The numbers on the diagonals from top left to bottom right should be 1, and the off-diagonal numbers should decrease moving away from the diagonal in any direction. If not, then repeat the matrix-making procedure starting with “To make the Dye Primer Matrix:” on page 6-8. **Note** The corresponding numbers for all three matrix files will be the same (or within 0.001 because of rounding).
4	Click Cancel.
5	Open or restart the Sequencing Analysis software and use dRhod_BigDye as the instrument file to analyze your sequencing data.

Making an Instrument File from a Sample File

An instrument file can be made from matrix standards as explained above, or it can be made from a sample file. This procedure requires fewer steps than running matrix standards, however, the matrix made from a sample file may not be as good as one made from matrix standards. The quality of a matrix file made from a sample file depends on the quality of the sample file used.

The best samples to choose for making a matrix have approximately 25% each of A, C, G, and T. A good example of this is the pGEM® control DNA that is included in every Applied Biosystems cycle sequencing kit. To create a matrix from a sample file, follow the steps below.

Step	Action
1	Before making the matrix, verify that lane tracking is accurate. Adjust if necessary.
2	Duplicate the unanalyzed sample file three times. Use the Duplicate command from the File menu in the Finder. You will have a total of four copies of the same sample file with the following names: ♦ Sample name ♦ Sample name Copy 1 ♦ Sample name Copy 2 ♦ Sample name Copy 3
3	These four sample files can now be used in the same way as the four matrix standard samples. The same instructions can be used with these four samples as with the four matrix standard samples.
4	Follow the directions in your instrument user's manual for creating an instrument file. Wherever the protocol indicates a specific matrix standard to be used, follow the table below:

Matrix Standard	Standard File
C…	Sample name
A…	Sample name Copy 1
G…	Sample name Copy 2
T…	Sample name Copy 3

Setting the Data Analysis Range

Overview Three values are important in setting the data analysis range:

- Peak 1 Location
- Start Point
- Stop Point

Peak 1 Location

The Peak 1 Location is the data point that marks the beginning of the first base peak in the data. This data point is the reference point for the spacing and mobility corrections performed by the basecalling software. Correct identification of this position is very important. If the Peak 1 Location value is wrong due to low signal, excess dye peaks, or other pathology, your data can show bad spacing or strange mobility shifts.

The Peak 1 Location value is calculated by the Sequencing Analysis software. In the 2.1 version of the software the term “Primer Peak Location” is used to designate this value.

Occasionally the basecalling software assigns the Peak 1 Location value either too early or too late. In either case, the correct Peak 1 Location should be identified and changed for optimal basecalling. The procedure used to change the Peak 1 Location (Primer Peak Location) will depend on which version of Sequencing Analysis software and the type of chemistry, *i.e.*, dye primer or dye terminator, you are using.

Table 6-5 below and Table 6-6 on page 6-16 show typical Peak 1 Location values for various instrument configurations and run conditions.

Table 6-5 Approximate Peak 1 Locations for the ABI 373 and ABI PRISM 377 DNA Sequencers

Instrument	Well-to-Read Length (cm)	Run Type	Gel Type[a]	Approximate Peak 1 Location (scan number)	Approximate Time to Peak 1 (min)
ABI 373[b]	24	Full or XL scan	6% Ac19	700	70
	24	BaseSprinter	4.75% Ac19	1000	50
	34	Full or XL scan	4.75% Ac19	800	80
	34	BaseSprinter	4.25% Ac19	1200	60
	48	Full or XL scan	4% Ac19	1000	100
ABI PRISM 377[c]	36	1200 scans/hr	4% Ac19, 4.5% Ac29, 5% LR, 4.8% PP	800	40
	36	2400 scans/hr	4% Ac19, 4.5% Ac29, 5% LR	800	20
	48	1200 scans/hr	4% Ac19, 4.25% Ac29, 4.75% LR, 5.25% PP	1200	60

a. Ac19 = 19:1 polyacrylamide, Ac29 = 29:1 polyacrylamide, LR = Long Ranger, PP = PAGE-PLUS.

b. ABI 373 and ABI 373 with XL Upgrade.

c. All models.

Note For the ABI 373 and ABI PRISM 377 instruments, scan numbers correspond to data points. These terms are sometimes used interchangeably.

Table 6-6 Approximate Peak 1 Locations for the ABI PRISM 310 Genetic Analyzer

Capillary Length (cm)	Run Type	Polymer Type	Approximate Peak 1 Location (data points)	Approximate Time to Peak 1 (min)
47	rapid sequencing	POP-6	1050–1150	25
61	long-read sequencing	POP-6	1700–2000	40

Note Peak 1 Locations and run times on the ABI PRISM 310 instrument can be affected by the laboratory temperature.

Start Point

The Start Point is the starting point for data analysis. The Start Point is normally the same as the Peak 1 Location value. However, the Start Point can be set later than the Peak 1 Location if desired (see page 7-64).

Note The Start Point can never be less than the Peak 1 Location value.

Stop Point

The Stop Point specifies the last raw data point to be included in the basecalling. If the default Stop Point is used, this endpoint is the last data point in the file. However, the basecalling can be stopped earlier if there is unusable raw data at the end of the file or if you want to analyze only a portion of the raw data in the file.

Peak 1 Location for Dye Primer Chemistries

The Peak 1 Location (Primer Peak Location) is the position of the first base peak. In dye primer chemistries this peak is found on the downward slope of the primer peak (Figure 6-1).

If you need to change the Peak 1 Location, use one of the following procedures depending on which version of the Sequencing Analysis software you are using (refer to Figure 6-1 during the procedure).

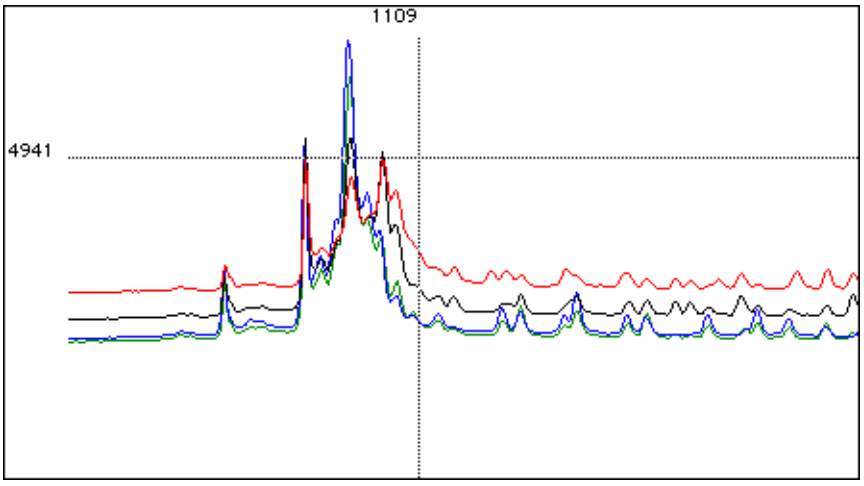


Figure 6-1 Electropherogram of raw data with the dashed vertical line showing the right edge of the primer peak, which is the recommended position of the Peak 1 Location for this sample file, *i.e.*, 1109 scans.

Using Sequencing Analysis Version 2.1

Step	Action
1	Launch the Sequencing Analysis software if it is not already open.
2	In the Sample File Queue display, double-click the first file to be analyzed to view the raw data. Zoom in completely. From the Window menu, choose Actual Size (or use the ⌘] keys on the keyboard).
3	Starting at the beginning of the raw data file, scroll along the data by clicking and holding the right direction arrow at the bottom of the window. Continue scrolling until the primer peak is approximately in the center of the window.
4	a. Click and drag in the window to change the cursor to a cross-hair. b. Move the cursor along the data until the vertical dotted line is aligned at the right edge of the primer peak (Figure 6-1 on page 6-16). c. Read the scan number (data point) that is reported at the top of the dialog box (1109 in the example shown in Figure 6-1). Use this number as the Primer Peak Location for the file.
5	Return to the Sample File Queue display. a. Highlight the name of the file just inspected and click the Custom Settings window. b. Select the Change Primer Peak check box. The Use Start Point and Change Primer Peak radio buttons become active. The Use Start Point radio button is selected by default, since the Primer Peak Location and the Start Point are the same in most cases. c. Click the Change Primer Peak radio button to display the entry field. The number in the data field is the number used for the Primer Peak Location during the last analysis. d. Enter the new number. **Note** If the value assigned for the Primer Peak location is greater than that assigned for the Start Point, the Start Point value needs to be changed to that of the Primer Peak Location. If you want the Primer Peak location to be used as the Start Point, changing the Start Point value and leaving the Change Primer Peak radio button as the default (Use Start Point) should also work. In Sequencing Analysis version 2.1, there is a link between the Start Point and the Primer Peak Location.

Using Sequencing Analysis Version 3.0 or 3.2

Step	Action
1	Launch the Sequencing Analysis software if it is not already open.
2	In the Sample Manager window, click the Add Files button and choose the sample to be analyzed. Click Done.
3	Highlight the sample name and click the Open Files button to display the raw data, or double-click the sample file name. Zoom in completely. From the Window menu, choose Actual Size (or use the ⌘] keys on the keyboard).

Using Sequencing Analysis Version 3.0 or 3.2 *(continued)*

Step	Action
4	Starting at the beginning of the raw data file, scroll along the data by clicking and holding the right direction arrow at the bottom of the window. Continue scrolling until the primer peak is approximately in the center of the dialog window.
5	a. Click and drag in the window to change the cursor to a cross-hair. b. Move the cursor along the data until the vertical dotted line is aligned at the right edge of the primer peak (Figure 6-1 on page 6-16). c. Read the scan number (data point) that is reported at the top of the dialog box (1109 in the example shown in Figure 6-1). Use this number as the Peak 1 Location of the file.
6	Return to the Sample Manager window. Highlight the Peak 1 Location box and enter the information. If you want to use the Peak 1 location value for the Start Point, enter the new Start Point as well. **Note** The Start Point value must be equal to or greater than the Peak 1 Location value or an error will occur. If the Start Point is not greater than the Peak 1 Location, highlight the Start Point box and enter a number greater than or equal to the number used for the Peak 1 Location.

Peak 1 Location for Dye Terminator Chemistries

As with dye primer chemistries, the Peak 1 Location value (Primer Peak Location) is the position that marks the beginning of the first base in the file. To determine the Peak 1 Location in terminator chemistry the software looks for an increase in signal followed by several peaks.

With dye terminator chemistries the raw data can show peaks between scan points. These peaks can be designated erroneously as the Peak 1 Location value by the software. These peaks are due to unincorporated dye terminators that are not removed during the purification of the dye terminator reactions.

If your data has excess dye peaks or if the software has not chosen the Peak 1 Location correctly, you will need to set this manually. To change the Peak 1 Location, use one of the following procedures depending on which version of the Sequencing Analysis software you are using (refer to Figure 6-2 on page 6-19 during the procedure).

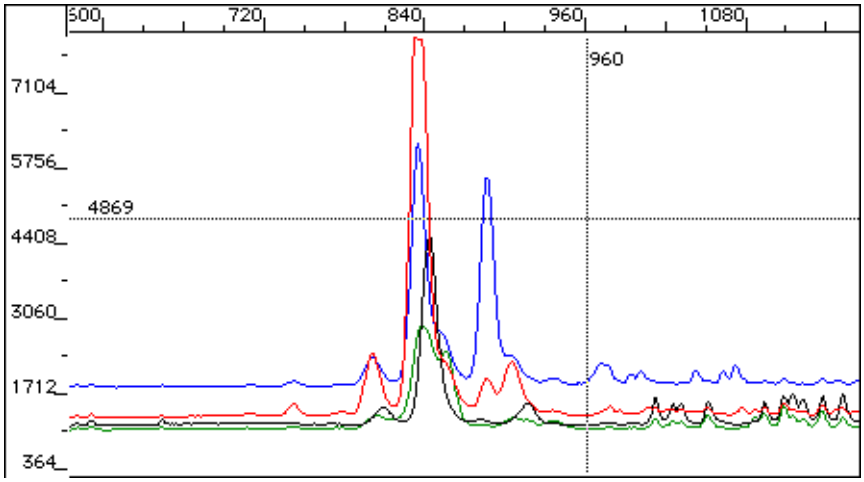


Figure 6-2 Electropherogram of raw data with the dashed vertical line showing the left edge of the first data peak, which is the recommended position of the Peak 1 Location for this sample file, *i.e.*, 960 scans.

Using Sequencing Analysis Version 2.1

Step	Action
1	Launch the Sequencing Analysis software if it is not already open.
2	In the Sample File Queue display, double-click the first file to be analyzed to view the raw data. Zoom in completely. From the Window menu, choose Actual Size (or use the ⌘] keys on the keyboard).
3	Starting at the beginning of the raw data file, scroll along the data by clicking and holding the right direction arrow at the bottom of the window. Continue scrolling until the fist data peak is approximately in the center of the window.
4	a. Click and drag in the window to change the cursor to a cross-hair. b. Move the cursor along the data until the vertical dotted line is aligned at the left edge of the first data peak (Figure 6-2). c. Read the scan number (data point) that is reported at the top of the dialog box (960 in the example shown in Figure 6-2). Use this number as the Primer Peak Location of the file.

Using Sequencing Analysis Version 2.1 *(continued)*

Step	Action
5	Return to the Sample File Queue display. a. Highlight the name of the file just inspected and click the Custom Settings window. b. Select the Change Primer Peak check box. The Use Start Point and Change Primer Peak radio buttons become active. The Use Start Point radio button is selected by default, since the Primer Peak Location and the Start Point are the same in most cases. c. Click the Change Primer Peak radio button to display the entry field. The number in the data field is the number used for the Primer Peak Location during the last analysis. d. Enter the new number. **Note** If the value assigned for the Primer Peak location is greater than that assigned for the Start Point, the Start Point value needs to be changed to that of the Primer Peak Location. If you want the Primer Peak location to be used as the Start Point, changing the Start Point value and leaving the Change Primer Peak radio button as the default (Use Start Point) should also work. In Sequencing Analysis version 2.1, there is a link between the Start Point and the Primer Peak Location.

Using Sequencing Analysis Version 3.0 or 3.2

Step	Action
1	Launch the Sequencing Analysis software if it is not already open.
2	In the Sample Manager window, click the Add Files button and choose the sample to be analyzed. Click Done.
3	Highlight the sample name and click the Open Files button to display the raw data, or double-click the sample file name. Zoom in completely. From the Window menu, choose Actual Size (or use the ⌘] keys on the keyboard).
4	Starting at the beginning of the raw data file, scroll along the data by clicking and holding the right direction arrow at the bottom of the window.
5	a. Click and drag in the window to change the cursor to a cross-hair. b. Move the cursor along the data until the vertical dotted line is aligned at the left edge of the first data peak (Figure 6-2 on page 6-19). c. Read the scan number (data point) that is reported at the top of the dialog box (960 in the example shown in Figure 6-2). Use this number as the Peak 1 Location of the file.
6	Return to the Sample Manager window. Highlight the Peak 1 Location box and enter the information. If you want to use the Peak 1 Location value for the Start Point, enter the new Start Point as well. **Note** The Start Point value must be equal to or greater than the Peak 1 Location value or an error will occur. If the Start Point is not greater than the Peak 1 Location, highlight the Start Point box and enter a number greater than or equal to the number used for the Peak 1 Location.

Stop Point

The Stop Point specifies the last raw data point to be including in the base calling. If the default Stop Point is used, this endpoint is the last data point in the file. Basecalling can be stopped earlier if there is clearly unusable raw data at the end of the file, or if you want to analyze only a portion of the raw data in the file.

Most often, this is done for short PCR products to eliminate the unusable data at the end of the run. With dye primer chemistries there will be a large peak at the end of the PCR product. Set the Stop Point just in front of this full-length PCR peak (Figure 6-3).

With dye terminator chemistries there is no full-length PCR peak, but there will be an abrupt end to sequence peaks in the data file. Set the Stop Point there.

For other samples, the Stop Point can be set where the data becomes too weak to give useful information or after a position where the sequence data stopped abruptly due to secondary structure in the template or for some other reason.

The inclusion of large amounts of data points with little or no signal at the end of the sequence will affect the scaling of the analyzed data. Their removal may be necessary to get better data in the region where there is good signal.

If you need to change the Stop Point, use the following procedure (refer to Figure 6-3 during the procedure).

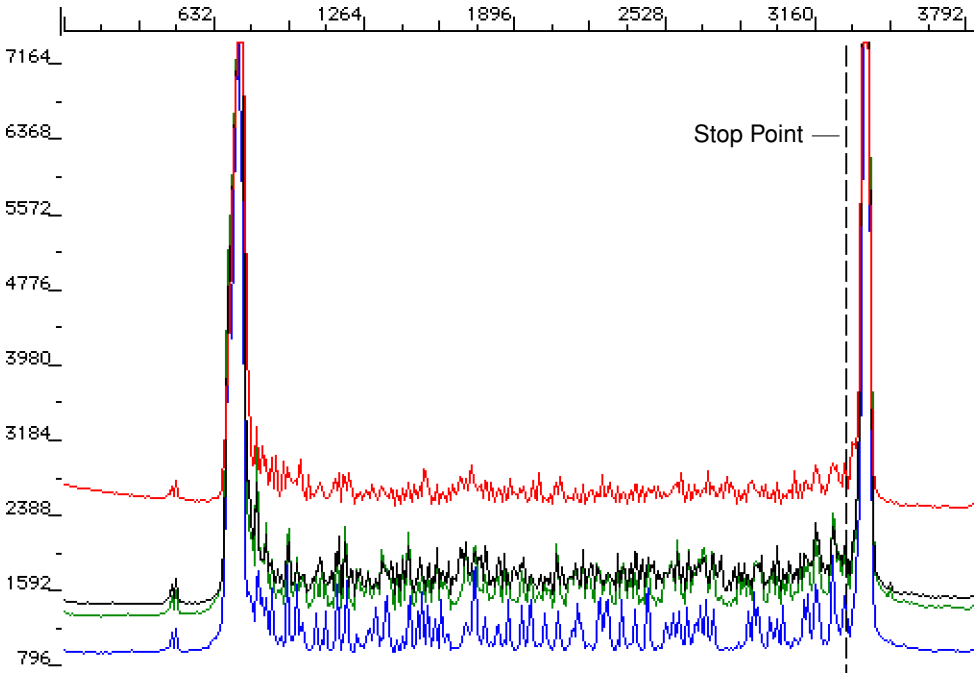


Figure 6-3 Electropherogram of raw data from a fluorescein/rhodamine dye primer sequencing reaction run on a short PCR product. The dashed vertical line shows the recommended position of the Stop Point.

Using Sequencing Analysis Version 2.1

Step	Action
1	Launch the Sequencing Analysis software if it is not already open.
2	In the Sample File Queue display, double-click the first file to be analyzed to view the raw data. Zoom in completely. From the Window menu, choose Actual Size (or use the ⌘] keys on the keyboard).
3	Starting at the beginning of the raw data file, scroll along the data by clicking and holding the right direction arrow at the bottom of the window.
4	a. Click and drag in the window to change the cursor to a cross-hair. b. Move the cursor along the raw data until the region of the desired Stop Point is in view. For a PCR product this would be in front of the full-length PCR peak (Figure 6-3 on page 6-21) or at the end of the sequence peaks. c. Align the cross-hair just before the peak. d. Read the scan number (data point) that is reported on the top of the window. Use this number as the Stop Point.
5	Return to the Sample File Queue display. a. Highlight the name of the file just inspected and click the Custom Settings window. b. Select the Sample file or files you wish to change. c. Click Custom Settings to display the Analysis Settings dialog box. d. Select the Change Stop Point check box. e. Enter a new number in the entry field.

Using Sequencing Analysis Version 3.0 or 3.2

Step	Action
1	Launch the Sequencing Analysis software if it is not already open.
2	In the Sample Manager window, click the Add Files button and choose the sample to be analyzed. Click Done.
3	Highlight the sample name and click the Open Files button to display the raw data, or double-click the sample file name. Zoom in completely. From the Window menu, choose Actual Size (or use the ⌘] keys on the keyboard).
4	Starting at the beginning of the raw data file, scroll along the data by clicking and holding the right direction arrow at the bottom of the window.
5	a. Click and drag in the window to change the cursor to a cross-hair. b. Move the cursor along the raw data until the region of the desired Stop Point is in view. For a PCR product this would be in front of the full-length PCR peak (Figure 6-3 on page 6-21) or at the end of the sequence peaks. c. Align the cross-hair just before the peak. d. Read the scan number (data point) that is reported on the top of the window. Use this number as the Stop Point.

Using Sequencing Analysis Version 3.0 or 3.2 *(continued)*

Step	Action
6	Return to the Sample Manager window. Highlight the Stop Point box and enter the information.

With the Sequencing Analysis software versions 3.0 and 3.2, you can set an earlier Stop Point either manually in the Sample Manager window, or automatically by changing the values on the Basecaller Settings page of the Preferences dialog box.

During basecalling, the basecaller considers both the Basecaller Settings in the Preferences and the Stop Point value in the Sample Manager window, and stops at the earliest designated endpoint.

Data Evaluation and Troubleshooting 7

Overview

In This Chapter

This chapter describes how to use the tools in the Sequencing Analysis software to evaluate and troubleshoot sequencing data. The chapter has three parts:

- Evaluating data using the information in the gel file and sample files (page 7-2)
- Practical examples of evaluating data (page 7-10)
- Troubleshooting sequencing data
  - DNA sequencing reactions (page 7-16)
  - DNA sequence composition (page 7-30)
  - Gel electrophoresis (page 7-44)
  - Capillary electrophoresis (page 7-55)
  - Software settings (page 7-62)

Data Evaluation

Introduction

There are many variables associated with DNA sequencing that can affect data quality. Understanding the data evaluation tools that are available in the Sequencing Analysis software can help in determining where problems may have occurred in the sequencing process. This section provides an introduction to these tools.

Gel Files

The gel file stores the raw data collected during the entire run of an ABI™ 373 or ABI PRISM® 377 instrument. Initially, the file contains the raw data collected during the run, a gel image, a copy of the data collection sample sheet, and a copy of the instrument file. After lane tracking and editing, the file also contains the lane tracking information and any changes made to the original information in the file.

Note The ABI PRISM® 310 Genetic Analyzer does not use gel files because samples are run consecutively in a capillary, not on a gel.

After the gel image has been generated and the lanes tracked, you should perform the following steps:

- Check the gel image for accurate lane tracking and assignment.
- If necessary, adjust the lane markers to correct lane numbering errors.
- If necessary, adjust the placement of the tracker lines.

Note The gel image can be magnified to aid in lane tracking. The gel contrast also can be adjusted to make the lanes easier to see if necessary.

- If you change any of the gel file or sample sheet information after extracting the sample file data, re-extract the data from the edited lanes to regenerate the information in the sample files.

It is helpful to look at the gel image, not only for correct lane assignment and tracking, but also for the following information:

- An overall impression of the run
  - Background noise in the gel
  - Buffer leaks
  - Instrument problems, such as a bad laser
  - Smeared or streaked lanes
  - Wavy lanes
  - Pinched lanes near beginning of run (bottom of gel image)
  - Band tilt (when the gel image is magnified)
  - Gel resolution (when the gel image is magnified)
- Excess dye peaks from dye terminator reactions
- Signal strength of individual samples
  - Failed reactions
  - Weak reactions

Figure 7-1 on page 7-4 shows a portion of a 96-lane gel with 52 lanes loaded. The samples include the following:

- Full-length plasmids sequenced with BigDye™ terminator chemistry (lanes 1–4)
- Short PCR products sequenced with BigDye terminator chemistry (lanes 5–26)
- Long and short PCR products sequenced with dRhodamine terminator chemistry (lanes 27–52, except 42 and 43)

Note Lanes 42 and 43 were not loaded because the wells were damaged.

This gel file has several problems, including the following:

- Background noise

  A horizontal yellow band runs across the loaded region of the gel. It is clearly visible in lanes 27–52. This band might show up as noise in the analyzed data: blue in dRhodamine chemistry and red in BigDye terminator chemistry (see "Dye/Base Relationships for Sequencing Chemistries" on page 2-14 and Figure 7-36 on page 7-29).

- Excess dye peaks

  Lanes 5–52 show excess dye peaks, which are more pronounced in lanes 5–26. The dye peaks result from poor ethanol precipitation. Figure 7-2 on page 7-4 shows a closeup view of the excess dye peaks at the bottom of the gel image. Using the closeup view can give information about peak shape as well as excess dye peaks. Refer to the *ABI PRISM DNA Sequencing Analysis Software User's Manual* for information on magnifying the gel image.

  Excess dye peaks obscure data at the beginning of the sequence.

- Weak and failed reactions

  Lanes 8–11, 18–22, 27, 29, 31, 33, and 48 have weak signal strengths that can cause the analyzed data to be noisy. Lanes 28, 30, 32, 34–47, and 49–52 show failed reactions, *i.e.*, they have signal too low to be analyzed or no usable data.

Observing the overall gel image or magnified areas of the gel image can help you identify features that can cause problems in data analysis.

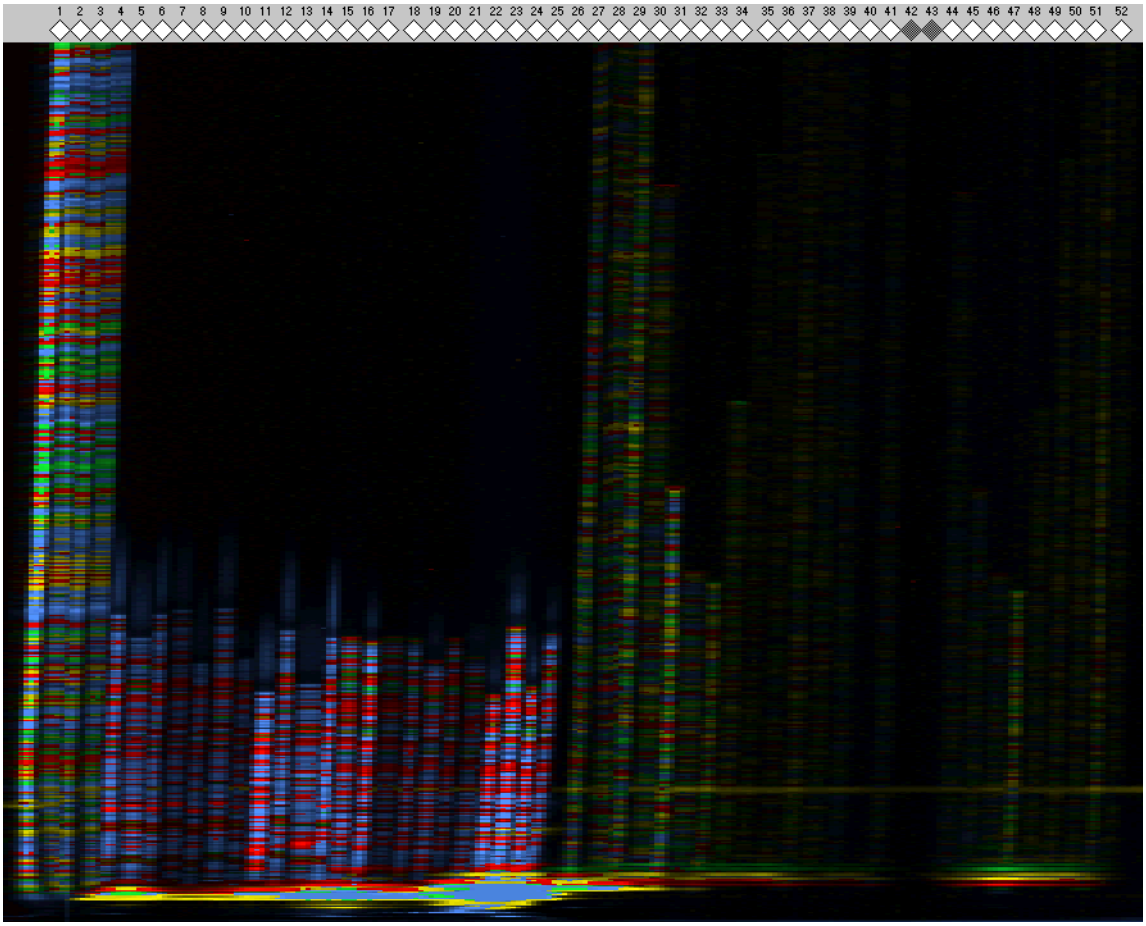


Figure 7-1 96-lane gel run on an ABI PRISM 377 DNA Sequencer at 1200 scans/hr

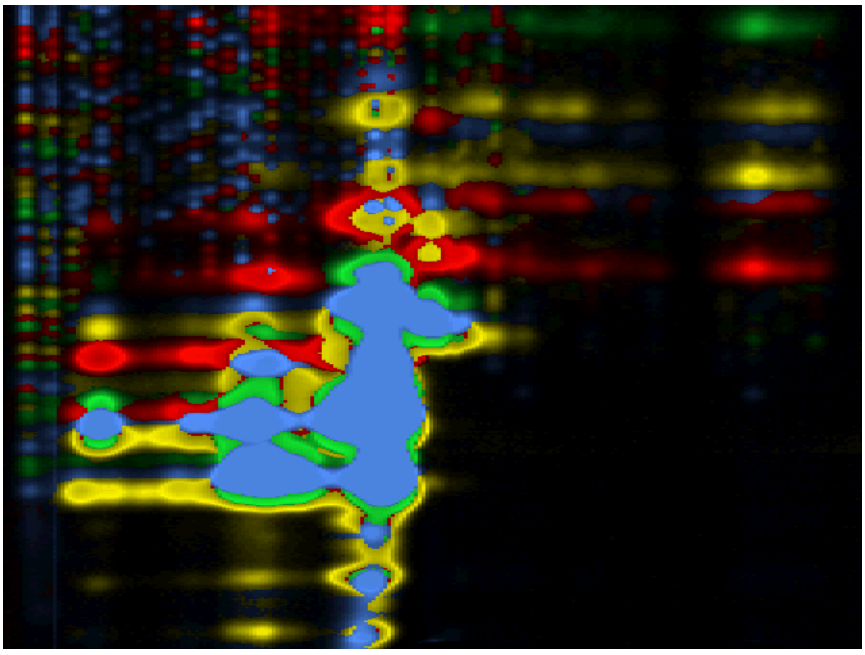


Figure 7-2 Zoomed-in view of the excess dye peaks in lanes 4–51 of Figure 7-1

Sample Files

There are six different fields within the sample file that can be used to display information about the sample. Three of these fields are useful for data evaluation:

- Electropherogram view
- Raw data view
- Annotation view

Refer to the *ABI PRISM DNA Sequencing Analysis Software User's Manual* for more information about the other three views and their uses.

For ABI PRISM 310 users, the sample file contains all of the necessary information for data evaluation.

Using the Electropherogram View

If your data was analyzed successfully, this is the default window that appears when opening the sample file. Scrolling through the data provides the following information for evaluating performance:

- Whether the Peak 1 Location and Start Point for data analysis are set correctly
- Presence of any extraneous dye peaks from unincorporated terminators or other fluorescent contaminants
- Peak shape and resolution
- Quality of mobility correction
- Match of basecalling with electropherogram peaks
- Point at which basecalling accuracy declines (number of Ns increases significantly)
- Signal-to-noise ratio
- Anomalies in the data that require further review

Figure 7-3 on page 7-6 shows an example of dRhodamine terminator data run on an ABI PRISM 377 DNA Sequencer. This sample was run in lane 29 of the gel shown in Figure 7-1 on page 7-4, so we already know that the sample has weak signal and excess dye peaks. The electropherogram view provides the following additional information:

- Excess dye peaks

  The dye peaks at the beginning of the sequence cause the Peak 1 Location and Start Point to be chosen incorrectly by the software (see page 7-64). The dye peaks also obscure data up to base 40.

- Poor peak shape and resolution

  The peaks in the data are broad and asymmetric. They are not well resolved from each other, which leads to miscalled bases. For example, the C peak at base 243 obscures the T peak at base 244, giving an ambiguous basecall (N).

- Poor signal-to-noise

  The data in the fourth panel is weak and noisy, causing many Ns.

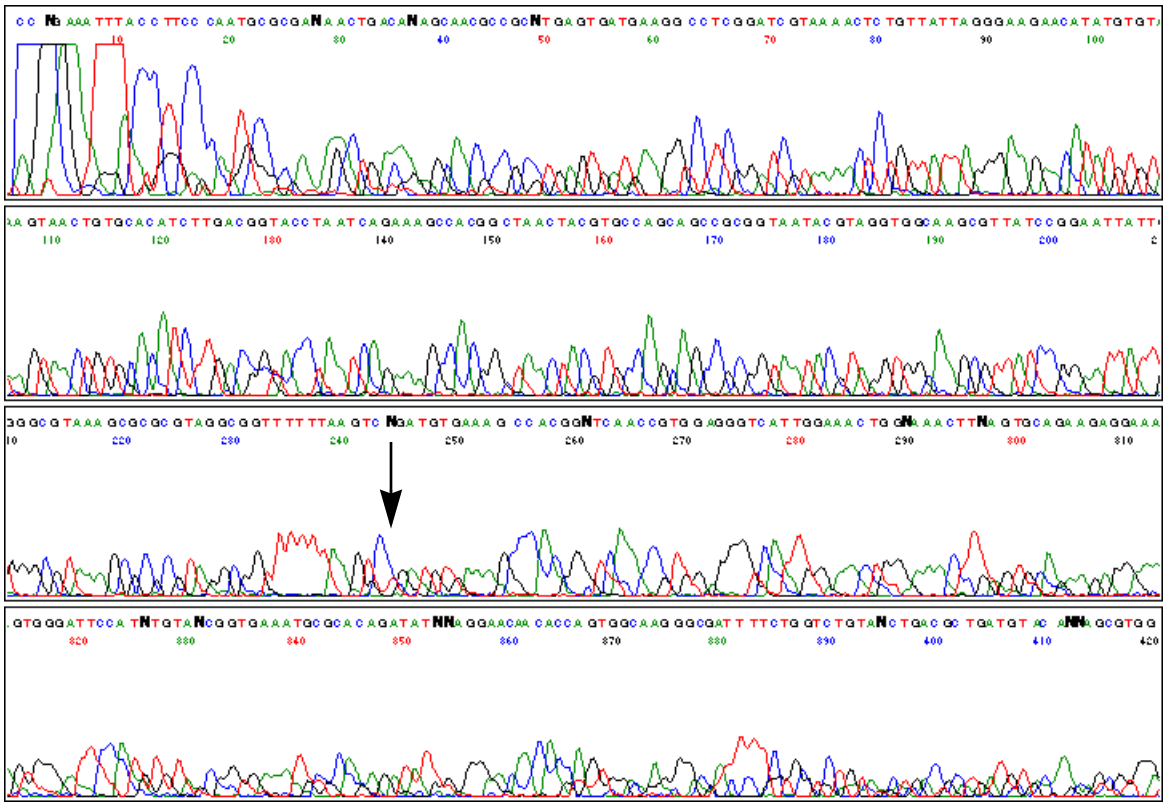


Figure 7-3 Electropherogram from the sample run in lane 29 of the gel in Figure 7-1 on page 7-4. The arrow points to the miscalled base at position 244 caused by poor resolution.

Using the Raw Data View

If the electropherogram is acceptable, it is not necessary to look at the raw data. However, looking at the raw data can be helpful in the following cases:

- The data has a poor signal-to-noise ratio
- The data has anomalies
- The data was not analyzed
- The Peak 1 Location and Start Point for data analysis were chosen incorrectly or a different Start Point is desired

The raw data view shows the following:

- Signal balance during the course of the run
- Abrupt signal changes during the run
- Elevated baselines
- Spikes (on the ABI PRISM 310 Genetic Analyzer)

Figure 7-4 on page 7-7 shows the raw data from lane 29 of the gel in Figure 7-1 on page 7-4. The raw data is weak and has excess dye peaks, which we already know from looking at the gel image and electropherogram.

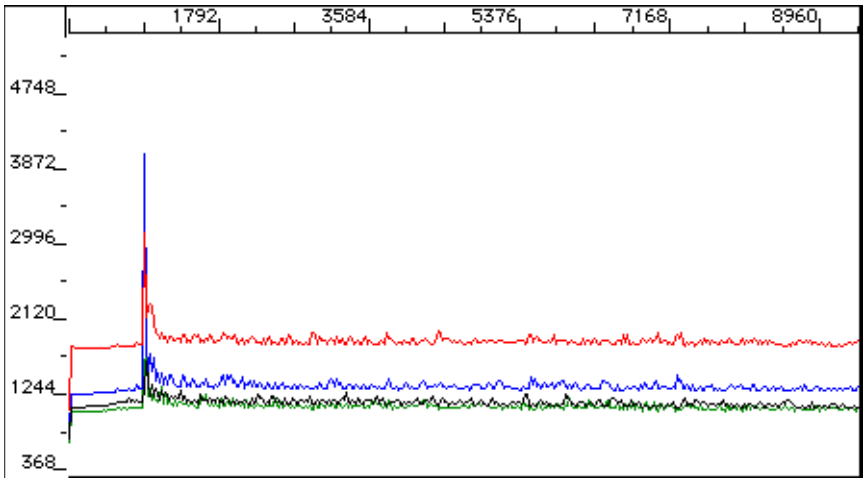


Figure 7-4 Raw data from the sample run in lane 29 of the gel in Figure 7-1 on page 7-4

If analysis fails, the raw data is the default window that appears when opening the sample file. Figure 7-5 shows a failed BigDye terminator reaction on the ABI PRISM 310 Genetic Analyzer. Only unincorporated dye terminator peaks are seen.

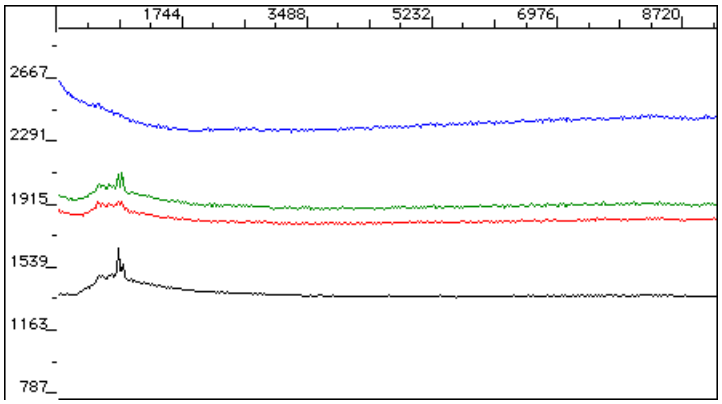


Figure 7-5 Raw data from a failed reaction

Figure 7-6 shows raw data from a successful BigDye terminator reaction on the ABI PRISM 310 Genetic Analyzer.

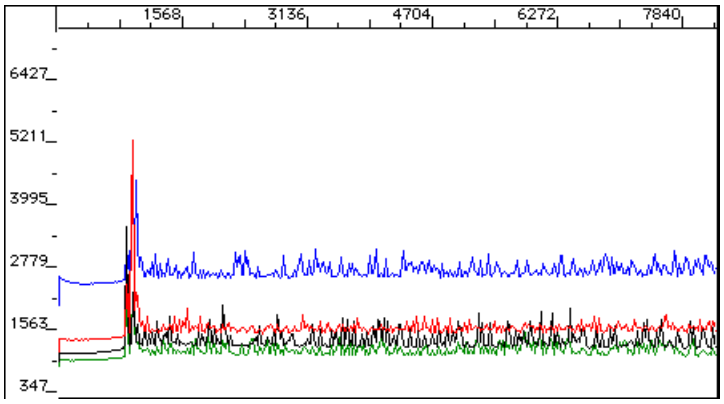


Figure 7-6 Raw data from a successful reaction

Figure 7-7 shows an example of BigDye terminator raw data from an ABI PRISM 377 DNA Sequencer run. The data shows signal imbalance. The signal is top heavy, *i.e.*, stronger at the beginning of the sequence, then tapering off.

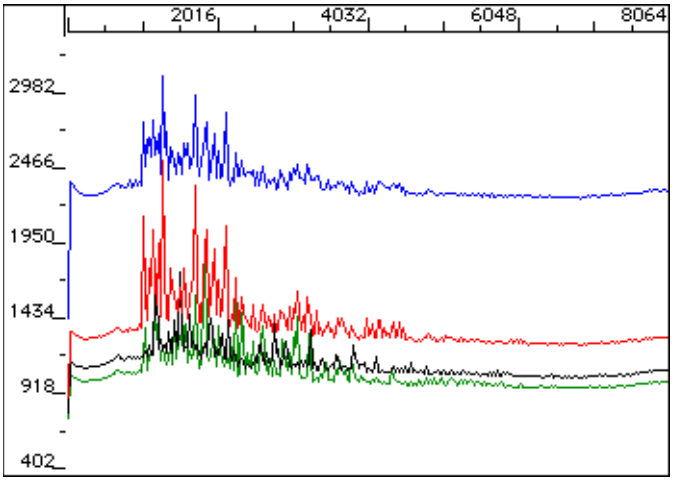


Figure 7-7 Top heavy data

Using the Annotation View

The annotation view shows data collection and analysis information associated with a sample file. The most useful information for evaluating data is the following:

- Dye set/primer (mobility) file
- Instrument (matrix) file
- Signal strength

  Signal strength indicators are a useful guide for determining whether signal strength is sufficient to obtain good data. Signal strengths for each nucleotide should usually be from 100–1000.

  – For fluorescein/rhodamine dye primers and rhodamine dye terminators, the signal strength for each base should be >100.

  – For BigDye™ primers, dRhodamine terminators, and BigDye terminators, the signal strength for each base should be >50. Signal limits are lower for these chemistries because of their better signal-to-noise ratios.

- Basecaller setting
- Base spacing

  The base spacing value indicates the speed of electrophoresis. The higher the base spacing, the slower the run, as there are more data points detected per peak. For basecalling, the software requires values between 8–16. If the base spacing falls outside this range, a default value is assigned (a red 9.0 in the Sample Manager in Sequencing Analysis version 3.0 and higher, and a -12 in Sequencing Analysis 2.1 and lower).

  Refer to the *ABI PRISM DNA Sequencing Analysis Software User's Manual* for a more detailed discussion of base spacing.

- Analysis Start Point and Peak 1 Location

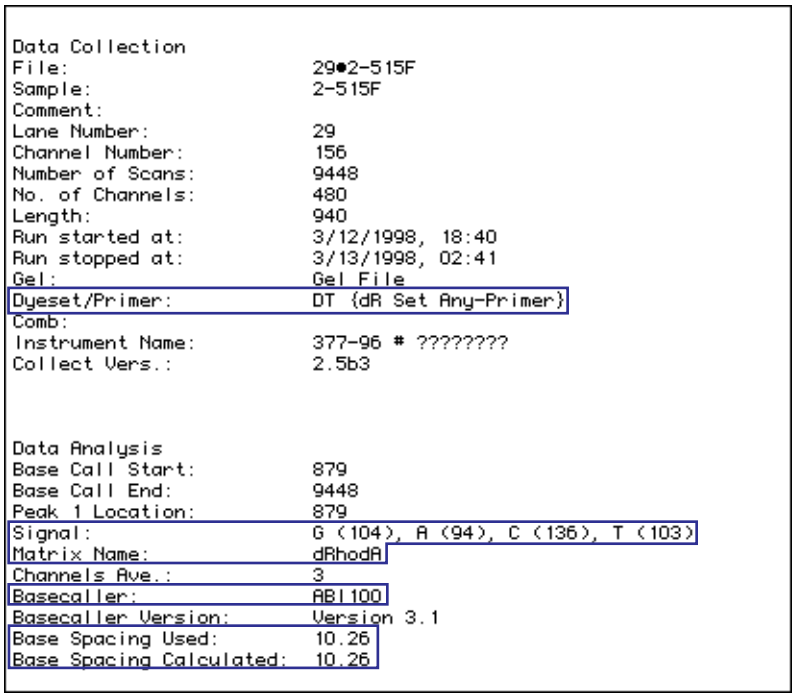
  These should be checked against those of other samples to determine whether data analysis started too early or too late.

- Run module (on the ABI PRISM 310 Genetic Analyzer)

  This is useful for determining whether the correct filter set was used to collect data.

Note All of the information described above can also be obtained from the top of the electropherogram printout.

Figure 7-8 shows the annotation view for lane 29 of the gel in Figure 7-1 on page 7-4. Useful items are highlighted.



```
Data Collection
File:                    29•2-515F
Sample:                  2-515F
Comment:
Lane Number:             29
Channel Number:          156
Number of Scans:         9448
No. of Channels:         480
Length:                  940
Run started at:          3/12/1998, 18:40
Run stopped at:          3/13/1998, 02:41
Gel:                     Gel File
Dyeset/Primer:           DT {dR Set Any-Primer}
Comb:
Instrument Name:         377-96 # ????????
Collect Vers.:           2.5b3


Data Analysis
Base Call Start:         879
Base Call End:           9448
Peak 1 Location:         879
Signal:                  G (104), A (94), C (136), T (103)
Matrix Name:             dRhodA
Channels Ave.:           3
Basecaller:              ABI100
Basecaller Version:      Version 3.1
Base Spacing Used:       10.26
Base Spacing Calculated: 10.26
```

Figure 7-8 Annotation view

In the annotation view for lane 29, the signal strength values are G: 104, A: 94, C: 136, and T: 103. These values are twice the minimum usually required for successful data analysis of dRhodamine terminator reactions, but are probably artificially raised by the excess dye peaks. The annotation view also shows that the correct dye set/primer file, instrument file, and basecaller were used.

Practical Examples of Data Evaluation

Overview

The following are common features of poor sequencing data:

- No recognizable sequence
- Noise
- Poor mobility correction
- Early signal loss

Examples of these problems are given below. Possible causes for them are listed. Some of the causes are described in more detail in the troubleshooting section of this chapter along with potential solutions.

No Usable Sequence

Figure 7-9 shows analyzed data with a high level of noise and lack of well-defined peaks. In this reaction, pGEM® control DNA was sequenced using the T3 primer. The pGEM control has no T3 annealing site.

For many failed reactions, analyzed data is not present because the signal strength is below the threshold for analysis. If excess dye peaks are present, they can raise signal levels artificially above this threshold. The data can be analyzed, but will not have usable sequence.

The raw data for this sample shows flat lines (Figure 7-5 on page 7-7). In the annotation view (not shown), signal strengths are very low (G:20, A:21, T:20, C:23). Base spacing for this sample is 8.93, but in failed reactions is often set to the default value.

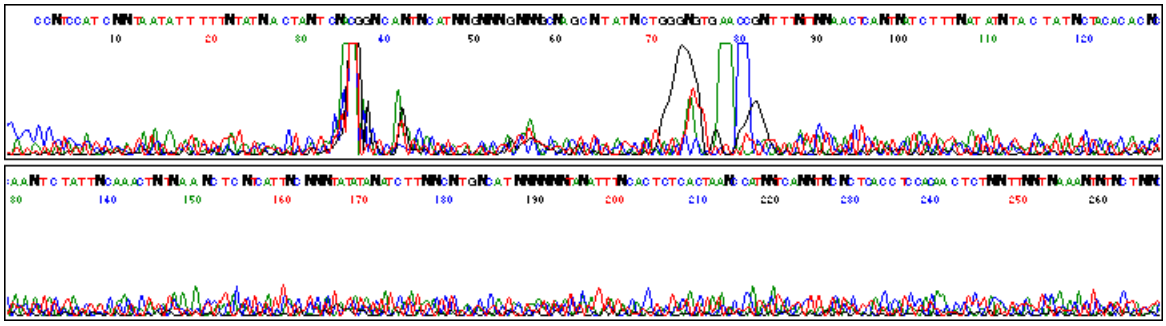


Figure 7-9 Analyzed data from a reaction that had very low signal due to the absence of a priming site in the template for the primer that was used

Possible causes of failed reactions:

- Primer has no annealing site, as in the example above
- Insufficient template
- Contaminated template
- Insufficient primer
- Poor primer design, *e.g.*, low melting temperature
- Old, mishandled, or missing reagents
- Thermal cycler failure
- Extension products lost during reaction cleanup

- Extension products not resuspended
- Lane tracking failure (ABI 373 and ABI PRISM 377 DNA Sequencers)
- Electrokinetic injection failure (ABI PRISM 310 Genetic Analyzer)

Noise

Some background noise is always present in sequencing data. Noisy data is characterized by a high background and peaks under peaks. Noise can be grouped into several categories, including the following:

- Noise throughout the sequence
- Noise up to or after a specific point in the sequence
- Noise caused by incorrect or poor quality instrument (matrix) file

Noise Throughout the Sequence

Figure 7-10 shows an example of noisy data. The background is high enough to cause ambiguities in basecalling, *e.g.*, the N at base 377. For this sample, the annotation view (not shown) indicates the cause to be low signal strengths (C: 47, A: 35, G: 30, T: 38). The raw data for this sample, shown in Figure 7-11, confirms the low signal strength. Other than the primer peak, the data is very weak.

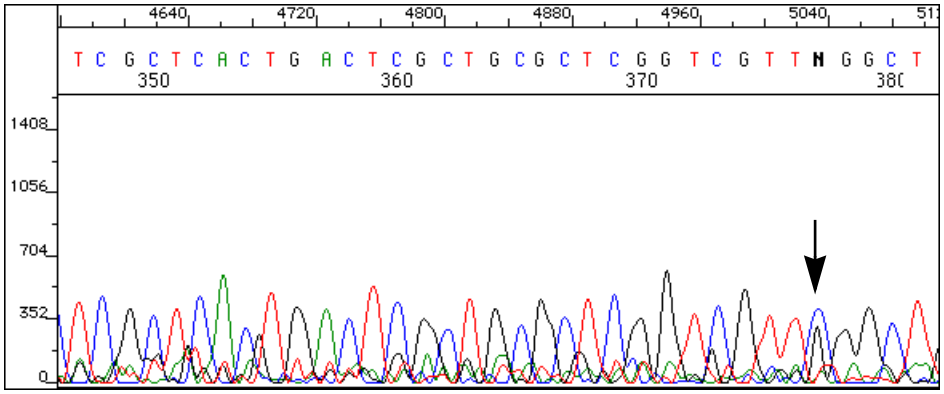


Figure 7-10 Analyzed data from a BigDye primer reaction run on an ABI PRISM 310 Genetic Analyzer

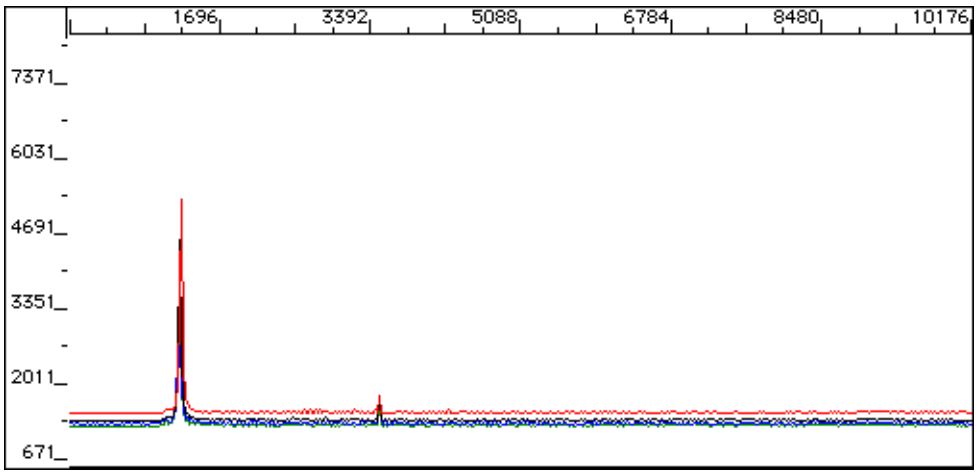


Figure 7-11 Raw data view for the sample file shown in Figure 7-10

Not all noisy data is caused by low signal. In other cases, the signal strength and the raw data can appear normal, so other possibilities should be considered.

Potential causes for noise throughout the sequence include the following:

- Low signal strength, as in the example above
- High signal strength, saturating the detector
- Contaminated template
- Expired or mishandled reagents
- Multiple templates in sequencing reaction
- Multiple priming sites
- Multiple primers
- N–1 primer
- Thermal cycler failure
- Lane tracking failure (ABI 373 and ABI PRISM 377 DNA Sequencers)
- Electrokinetic injection problem (ABI PRISM 310 Genetic Analyzer)
- Incorrect run module used to collect the data
- Incorrect instrument (matrix) file used to analyze the data

Noise Up To or After a Specific Point in the Sequence

Figure 7-12 shows data from a plasmid clone sequenced with the BigDye terminators on an ABI PRISM 377 DNA Sequencer. Because the noise starts after the multiple cloning region of the vector (base 62, see arrow below), the probable cause was picking a colony that was not well isolated and also had bacteria with no insert or a different insert in the plasmid.

The raw data (not shown) appears normal. For this sample, the annotation view shows normal base spacing. In other cases, it could show default base spacing if the noise occurred at the beginning of the sequence, because base spacing is calculated from the early data.

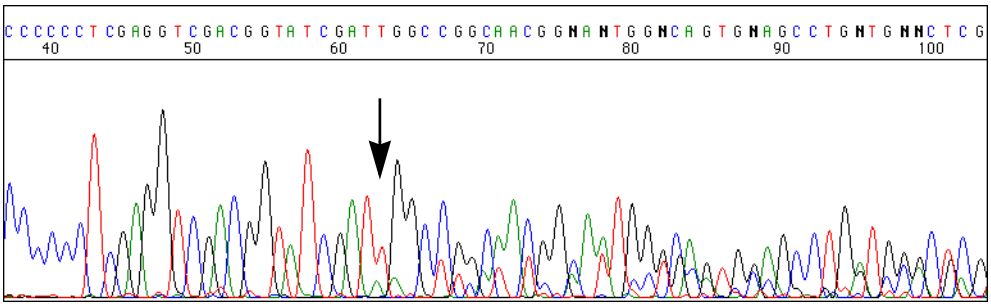


Figure 7-12 Noisy data after a specific point

This kind of noise can have the following causes:

- Mixed plasmid or PCR preparation, as in the example above
- Frame shift mutation
- Primer-dimer contamination in PCR sequencing
- Slippage after homopolymer region in template

Noise Caused by an Incorrect or Poor Quality Instrument (Matrix) File

Figure 7-13 on page 7-13 shows BigDye terminator data, collected on an ABI PRISM 377 DNA Sequencer, with specific peaks under peaks throughout the run. For example, every black peak has a smaller red peak underneath it.

The annotation view (not shown) indicates that an incorrect instrument file, one for Filter Set A instead of Filter Set E, was used to analyze the data. If your data looks like this, you should check that the correct run module was used to collect the data. If the correct run module and instrument file were used, you may have a poor quality matrix. In this case, the instrument file should be remade (see page 6-7).

For this type of noise, the raw data appears normal because a matrix is not applied to raw data.

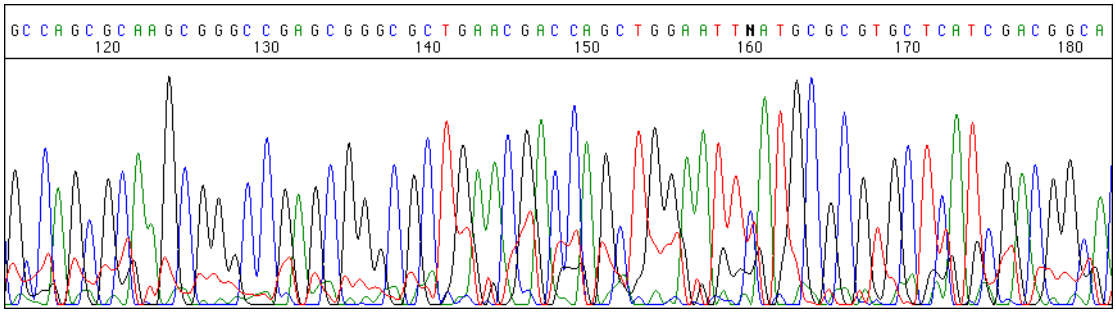


Figure 7-13 BigDye terminator data analyzed with an incorrect (Filter Set A) instrument file

Potential causes of this type of noise are the following:

- Incorrect instrument file used, as in the example above
- Poor quality instrument file
- Choosing the wrong run module, which causes the wrong filter set to be used to collect the data

Poor Mobility Correction

Figure 7-14 shows BigDye terminator sequencing data with poor mobility correction. Some peaks are very close together, while others have large gaps between them. The annotation view (not shown) reveals that the BigDye primer mobility file was used to analyze the data.

Looking at the raw data is not helpful because mobility corrections are not applied to raw data.

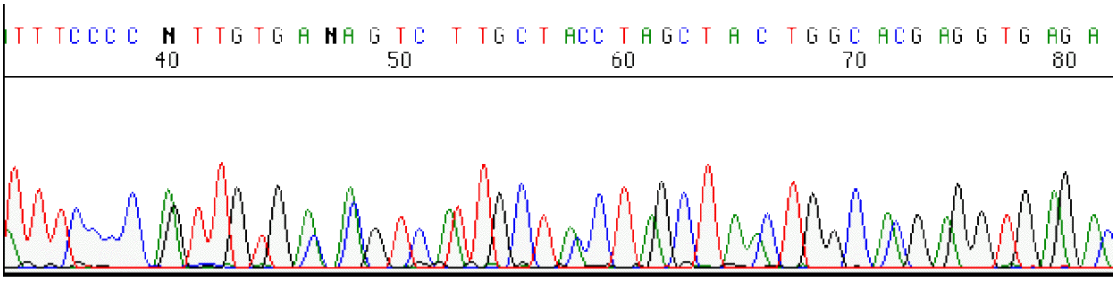


Figure 7-14 BigDye terminator data analyzed with a BigDye primer dye set/primer (mobility) file, which results in poor mobility correction

There are three potential causes of poor mobility correction:

- Choosing the incorrect dye set/primer (mobility) file, as in the example above
- Incorrect Peak 1 Location for data analysis

  If the Peak 1 Location is not set correctly, the mobility algorithm is not applied correctly (see “Setting the Data Analysis Range” on page 6-15).
- Using a gel matrix on the ABI 373 or ABI PRISM 377 DNA Sequencer with very different separation properties from the gel matrices that were used to construct the dye set/primer (mobility) files

  For information on selecting the correct dye set/primer file for your gel type, refer to the *ABI PRISM DNA Sequencing Analysis Software User's Manual*.

Early Signal Loss

There are two kinds of early signal loss:

- Gradual
- Abrupt

Gradual Signal Loss

Figure 7-15 shows BigDye terminator sequencing data where the signal gradually dies after a region of CTT trinucleotide repeats starting at base 280. In this example, the loss of signal is enough to cause some miscalls, but there is still usable data. Bases were called accurately to 650. Figure 7-16 on page 7-15 shows raw data from the same sample. As in the analyzed data, the signal in the raw data dies out gradually in the repeat region.

In this example, the annotation view does not provide useful information. If the signal loss had occurred early in the sequence, signal strengths would be low and default base spacings might be assigned.

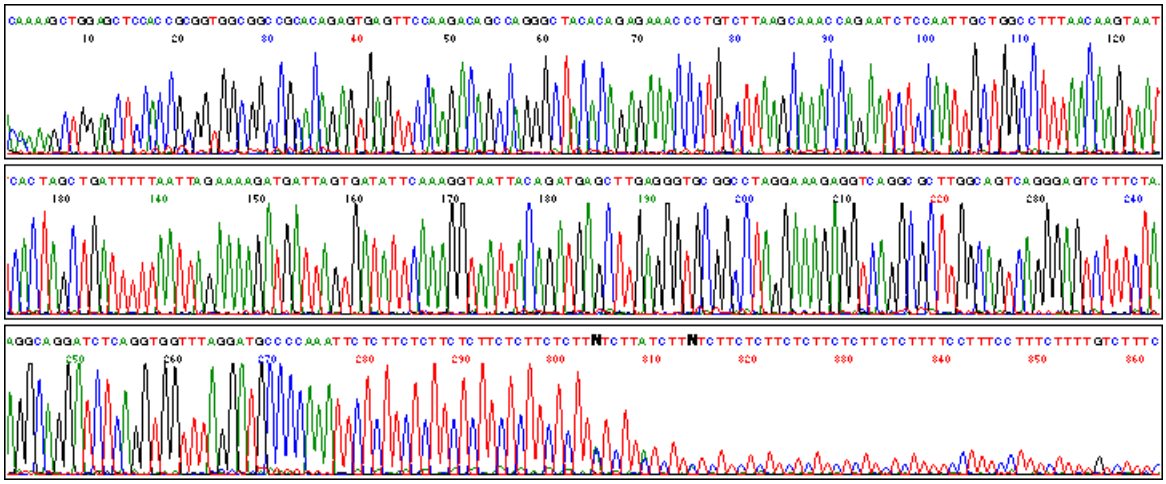


Figure 7-15 Gradual signal loss

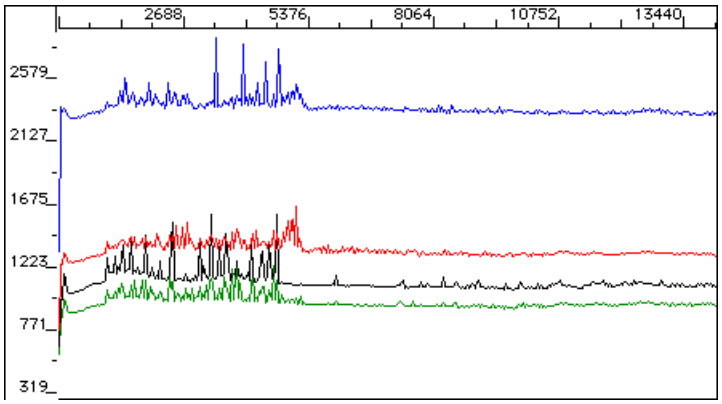


Figure 7-16 Raw data view from the sample shown in Figure 7-15 on page 7-14

Abrupt Signal Loss

Figure 7-17 shows BigDye terminator sequencing data that has an abrupt loss of signal. The signal dies out so completely that the basecaller fails after base 640.

The raw data view in this example looks like that in Figure 7-16, but with flatter lines after the signal loss. The annotation view would be uninformative, as it was in the case of gradual signal loss.

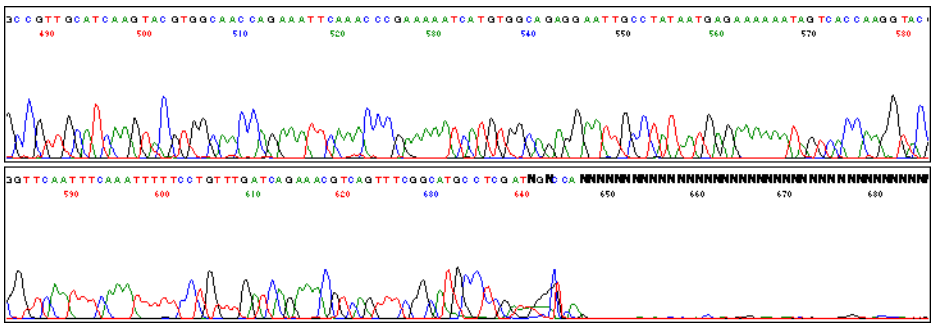


Figure 7-17 Abrupt signal loss

Gradual and abrupt signal losses have many causes, including the following:

- Region of secondary structure in the template
- Template sequence idiosyncrasies
- Poor lane tracking, such that the tracker line diverges from the data
- Poor quantitation of primer and/or template, leading to top heavy data
- Buffer leak on the ABI 373 or ABI PRISM 377 DNA Sequencer

Troubleshooting Sequencing Reactions

Overview

This section shows common examples of unsatisfactory sequencing data caused by poor template preparation, sequencing reaction setup, or reaction cleanup procedures. Refer to the table on page 7-39 for a more complete guide to troubleshooting sequencing data.

Poor Template Quality

Poor template quality is one of the most common causes of sequencing problems (see "DNA Template Quality" on page 3-15). Template quality can be affected by:

- Residual salts or organic chemicals carried over from the template preparation
- Incomplete removal of cellular components such as RNA, proteins, polysaccharides, and contaminating chromosomal DNA

  The presence of residual RNA or chromosomal DNA in the template preparation will affect the quantitation of the DNA if this is done spectrophotometrically. The presence of such contaminants can be determined by analysis of the template preparations on agarose gels (see "Determining DNA Quality" on page 3-16).
- Degradation of DNA in storage
- More than one template DNA in the sequencing reaction

The appearance of the sequence data will vary depending on the source of the problem.

Contaminants

The presence of various types of contaminants in the template preparation can result in inhibition of the sequencing reaction, giving weak signal. This may or may not be accompanied by significant noise.

Figure 7-18 on page 7-17 shows data from a template preparation that gave fairly clean data with BigDye terminator chemistry, but with weak signal. Although the sequence data is fairly good, the background is apparent because of the low signal and the increased scaling of the noise by the software. Increasing the amount of template from 0.25 µg to 1.0 µg resulted in only a slight increase in total signal strength, from 144 to 189.

The template was precipitated with ethanol and resequenced (Figure 7-19 on page 7-17). After ethanol precipitation the signal was much stronger, with 0.25 µg having signal strength of 558 and 1.0 µg having signal strength of 1383. The ethanol precipitation probably removed a contaminant that was present in the original template preparation and was inhibiting the sequencing reaction (see "Salt Contamination" on page 7-25).

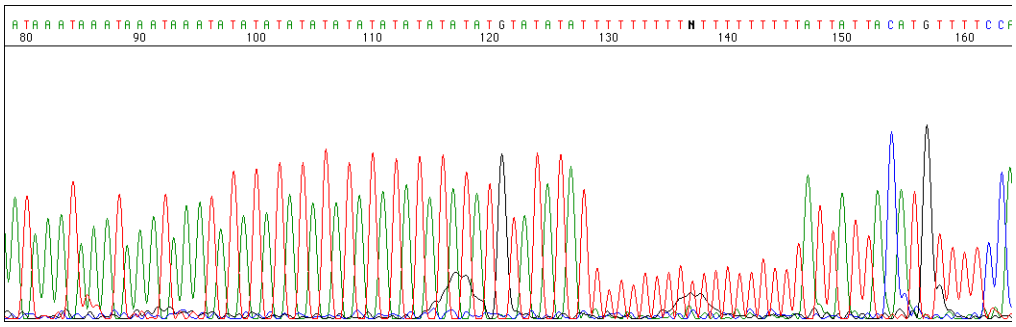


Figure 7-18 Sequence data obtained from a contaminated template preparation. 0.25 µg of template was sequenced. The total signal strength was 144.

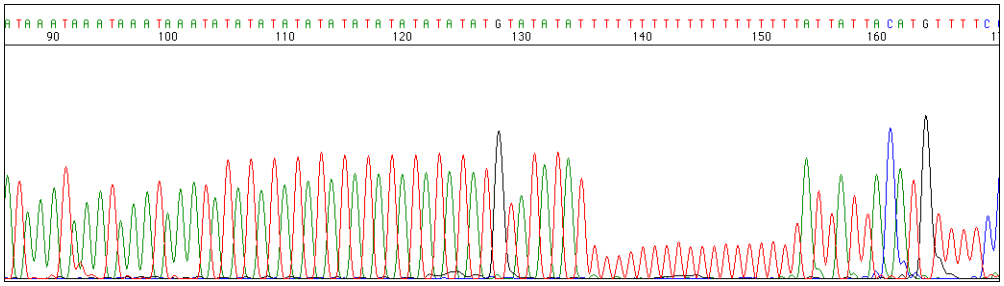


Figure 7-19 Same template after ethanol precipitation. 0.25 µg of template was sequenced. The total signal strength was 558.

Degraded DNA

Nuclease contamination in a template preparation and repeat freeze-thaw cycles can result in degradation of DNA over time. Figure 7-20 shows sequence data from a BigDye primer reaction done with an old template preparation. There is a large stop peak present in the sequence data after base 320.

Note Stop peaks are common to all chemistries, but are detected only in dye primer chemistries (see page 7-30).

When the DNA was reisolated and the new template DNA sequenced, the data was clean in this region (Figure 7-21 on page 7-18).

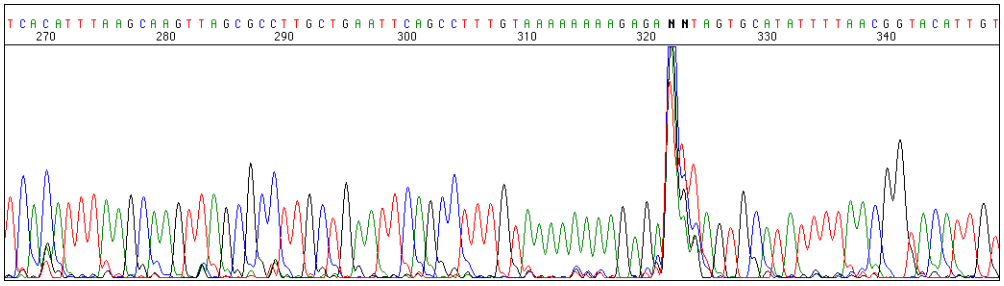


Figure 7-20 Sequence data obtained using BigDye primers with an old template preparation

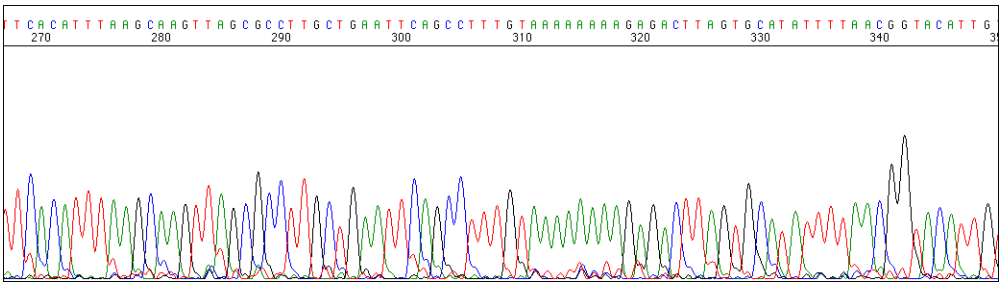


Figure 7-21 Sequence data obtained using BigDye primers with a newer template preparation

Multiple Templates

The presence of more than one template in a reaction will result in multiple, overlapping sequences in the data. This can happen with both PCR templates and cloned DNA templates. For PCR reactions, lack of complete specificity can result in more than one product being produced (Figure 7-22).

The majority of cleanup procedures for PCR products are designed to remove unincorporated nucleotides and residual PCR primers, not secondary PCR products. The presence of secondary PCR products can be detected by agarose gel electrophoresis (see page 3-16).

Optimization of PCR conditions and/or use of a Hot Start method usually result in generation of a clean PCR product. If necessary, the PCR product can be gel purified before sequencing.

The sequence data shown in Figure 7-22 is from a PCR product that showed three bands on an agarose gel, even though the PCR product was cleaned up by ultrafiltration. After gel purification, good sequence data was obtained for the product of interest (Figure 7-23).

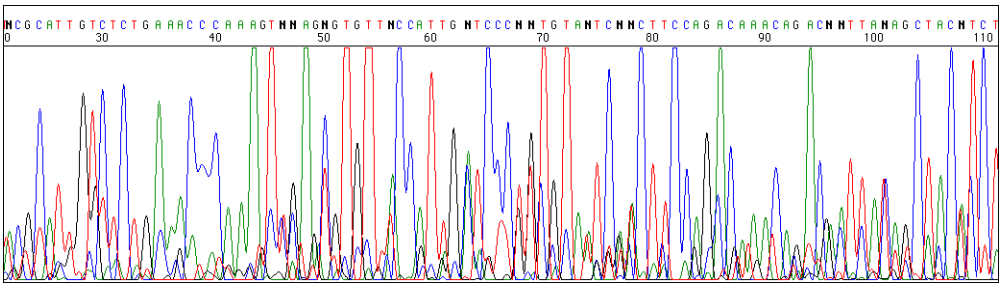


Figure 7-22 Rhodamine dye terminator data from a contaminated PCR product

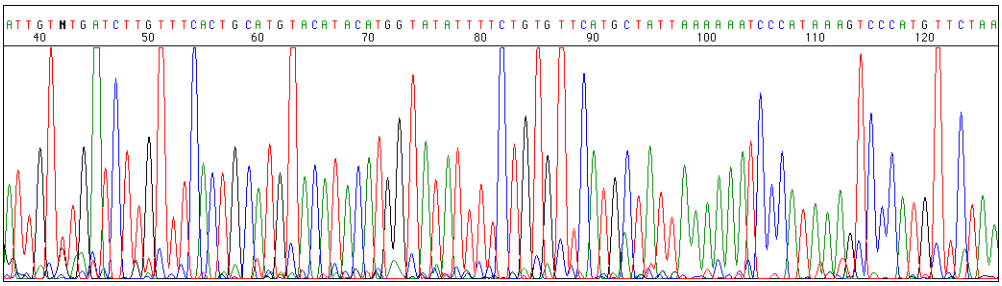


Figure 7-23 Rhodamine dye terminator data from the same PCR product after gel purification

For cloned DNA the presence of two or more sequences generally results when mixed plaques or colonies are picked. In Figure 7-24 the plasmid DNA used in the sequencing reaction was isolated from a mixed culture that contained bacteria with only vector DNA and bacteria with vector containing the insert of interest. With a mixed clone such as this, the early sequence data is clean because this is the sequence of the multiple cloning site. After the cloning site the data is noisy, with peaks under peaks.

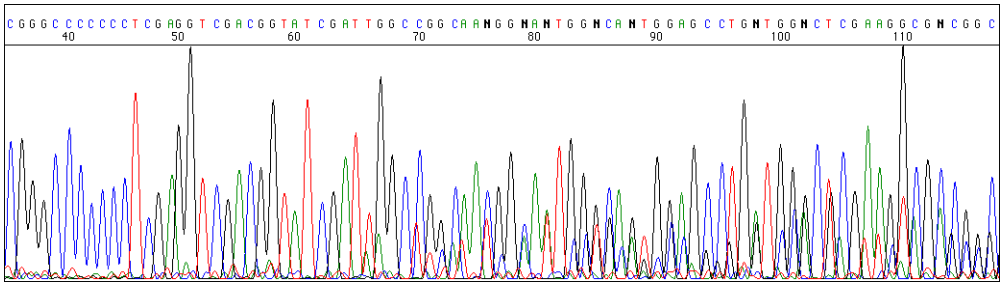


Figure 7-24 Sequence data obtained with BigDye Terminators and a template preparation that contained two different plasmid templates

When the DNA was reisolated from a pure colony, clean sequence data was obtained (Figure 7-25). When picking bacterial colonies for growth and DNA isolation select a colony that is well isolated. With M13 plaques, fresh plates should be used for plaque picking. Check the DNA template purity on an agarose gel (see page 3-16).

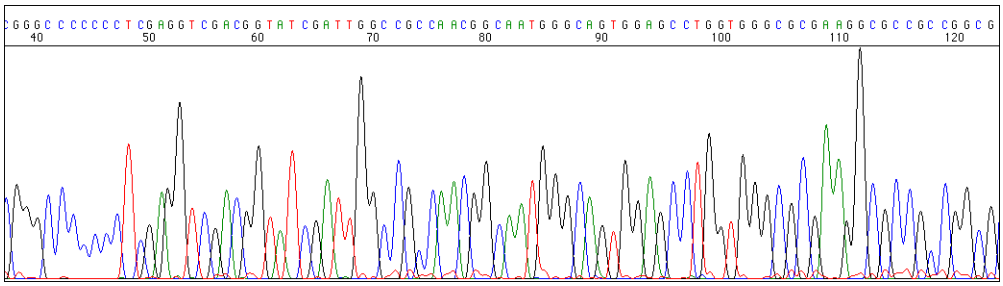


Figure 7-25 Sequence data obtained with BigDye Terminators and a template isolated from a pure colony

Primer-Related Problems

There are several primer-related problems that can affect the data obtained in sequencing reactions. These can be divided into three categories based on the type of problem seen in the data:

- No recognizable sequence
- Very weak signal
- Two or more sequences present in the electropherogram

No Recognizable Sequence

If there is no priming site for the primer in the template, no sequence data will be obtained. The raw data will show only a flat line except for the primer peak in primer reactions or sometimes excess terminator peaks in the case of terminator reactions (Figure 7-9 on page 7-10).

This would also happen if the wrong primer is used for a particular vector, or if a mutation is present in the primer binding site in the vector that results in the primer not working effectively.

Such a mutation is present in some samples of pUC18 and vectors such as pUC118 that were derived from it (Lobet *et al.*, 1989). These vectors are missing a C in the *lacZ* region that is complementary to the 3´ base for some reverse sequencing primers.

Very Weak Signal

The signal in sequencing reactions can be very weak if the primer anneals poorly because of a low melting temperature (T_m). Generally primers should have a T_m above 45 °C (see“Primer Design” on page 3-18). In some cases, however, lowering the annealing temperature in the reaction can help in obtaining good signal.

The primer used to generate the sequence data in Figure 7-26 was 15 bases long and had a T_m of 41 °C. The same template was resequenced with a 30-mer primer that had a melting temperature of 58 °C (Figure 7-27).

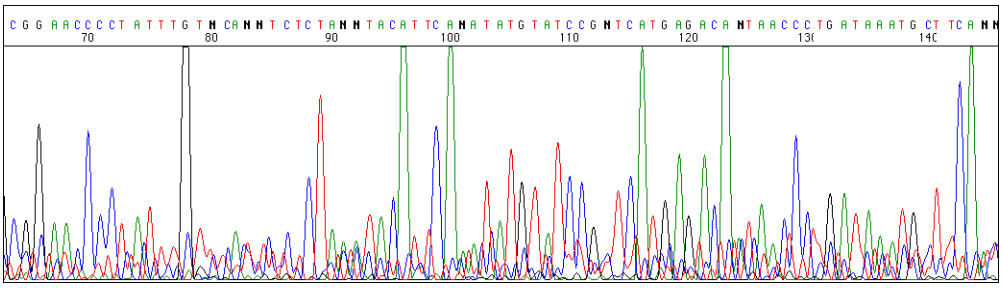


Figure 7-26 Rhodamine dye terminator data using a 15-mer primer and AmpliTaq® DNA Polymerase

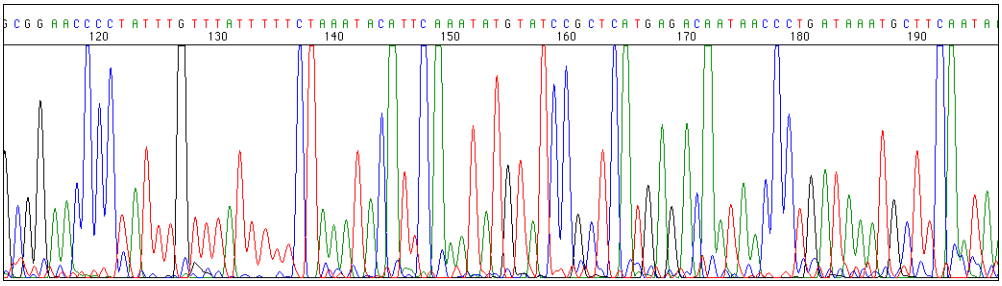


Figure 7-27 Rhodamine dye terminator data from the same template using a 30-mer primer and AmpliTaq DNA Polymerase

Weak signal can also result if the primer anneals poorly due to secondary structure, particularly at the 3´ end. Whether or not a primer is likely to have significant secondary structure can be determined by analyzing its sequence with one of several primer design programs that are available, such as Primer Express™ software (P/N 402089).

Two or More Sequences Present

More than one sequence can be present in an electropherogram if:

- The primer used in the reaction is contaminated with N–1 primer (primer that is one base shorter than the desired primer).
- More than one primer is present in the sequencing reaction.
- There is a secondary hybridization site for the primer in the template.

In each case, there will be two or more sequences in the electropherogram. The data will look similar to that shown in Figure 7-28, with peaks under peaks except where the bases in both sequences are the same.

Figure 7-28 shows data from a sequencing reaction where the primer was contaminated with N–1 primer. Careful examination of the data from such a reaction shows that the two sequences are identical, except that the sequence from the N–1 primer is one base shorter than the sequence from the full length primer. For example, the G at base 63 is shadowed by a smaller G underneath the A at base 62 (see arrow). No additional peak is seen at position 61 because the extension products from both the full length primer and the N–1 primer have an A at this position.

N–1 sequence can be detected in a sequencing reaction if the N–1 primer is present at the level of 5–10% of the correct primer concentration. However, somewhat higher levels can be tolerated, depending on the particular chemistry used.

In the sequence data shown in Figure 7-28, the contamination by N–1 primer is 40%. This results in ambiguities (Ns) in the basecalling. In a high quality synthesis, the N–1 contamination should be slight. The concentration of N–1 sequence in the primer can be further reduced by HPLC purification.

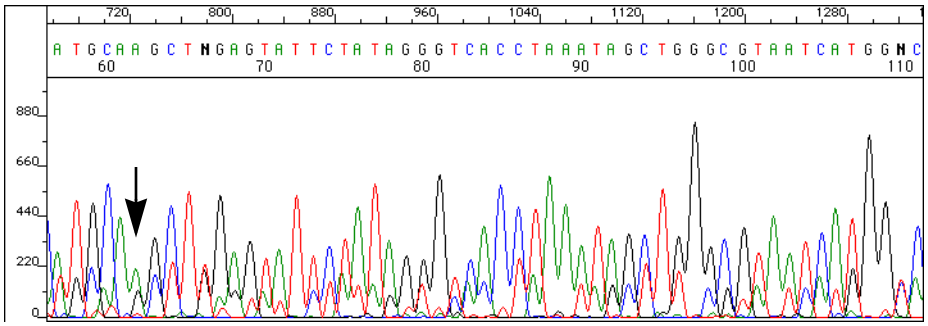


Figure 7-28 pGEM control DNA sequenced with BigDye terminator chemistry and –21 M13 primer contaminated by 40% N–1 primer

The presence of more than one primer in a sequencing reaction can be a problem when sequencing PCR products. Since two primers are present in the PCR reaction, failure to completely remove the unincorporated primers from the PCR product will result in the carryover of some of these primers into the sequencing reaction.

When using dye primer chemistries, fragments that extend from these residual PCR primers will be unlabeled. If the concentration of these fragments is not too high, there should not be a significant impact on the reaction. With dye terminator chemistries, however, the extension products from the residual primers will also be labeled and will result in a second sequence being present in the data.

Complete removal of the unincorporated primers from the PCR amplification before sequencing will prevent this. Ultrafiltration in a Centricon®-100 column is an effective way to remove the unincorporated primers as well as the unused dNTPs (see page 3-12).

If a secondary hybridization site for the primer is present in the template, two sequences will be detected, resulting in noisy data.

Hybridization at a secondary site that is not a perfect match for the primer will happen more readily at lower annealing temperatures and at higher concentrations of primer. To minimize this, keep the annealing temperature as high as possible and do not use excessively high concentrations of primer.

If the secondary hybridization site is a perfect match for the primer due to the presence of homologous sequence, you will have to redesign the primer or use a different sequencing strategy.

Pull-up Peaks

Figure 7-29 shows rhodamine dye terminator data collected on an ABI PRISM 377 DNA Sequencer. The data has pull-up peaks (also known as bleedthrough), multiple peaks in the same position at some points.

Pull-up peaks are caused by very strong signals that saturate the detector. These signals are therefore clipped digitally (truncated). The software underestimates the amount of signal at these positions, so it also underestimates the amount of spectral overlap to correct. Hence pull-up peaks are seen.

These extra peaks are consistently observed at places in the electropherogram where there is a tall peak. Usually, they are of only one color. Occasionally, when the signals are very strong, more than one pull-up peak color is observed, as in Figure 7-29.

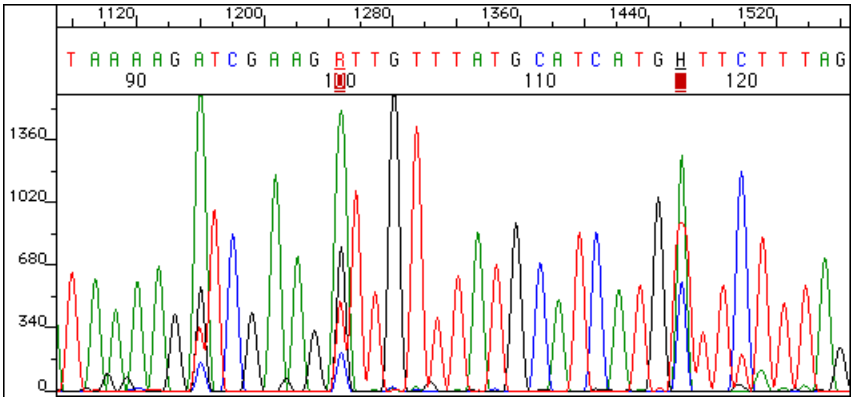


Figure 7-29 Pull-up peaks with rhodamine dye terminator chemistry

In the sample file from Figure 7-29, the total signal strength shown in the annotation view is 6077, which is much higher than the recommended maximum of 4000 (1000 for each base).

You can also look in the raw data to determine if signals are too strong. After zooming in, if any peaks are truncated at the top (*i.e.*, off-scale) then pull-up peaks may be observed in the analyzed data.

Very strong signals are common when sequencing short PCR fragments, as the sequencing reaction is often very efficient. You may need to load less of this type of sample to compensate for the increased signal.

Another influence on pull-up peaks is the sequence-specific peak patterns associated with each chemistry. The rhodamine dye terminators have several peak patterns in the electropherogram data where very strong signals occur. For example, an A after G, a T after G, and a C after one or more Ts all display an enhanced signal. These base composition effects increase the chance of a pull-up peak being observed at these positions (Figure 7-29 on page 7-22).

The dRhodamine terminators and BigDye terminators have more uniform peak heights that lessen the potential for individual signals to go off-scale (Figure 7-30). However, if combined signals are above 4000, then pull-up peaks are likely to be observed.

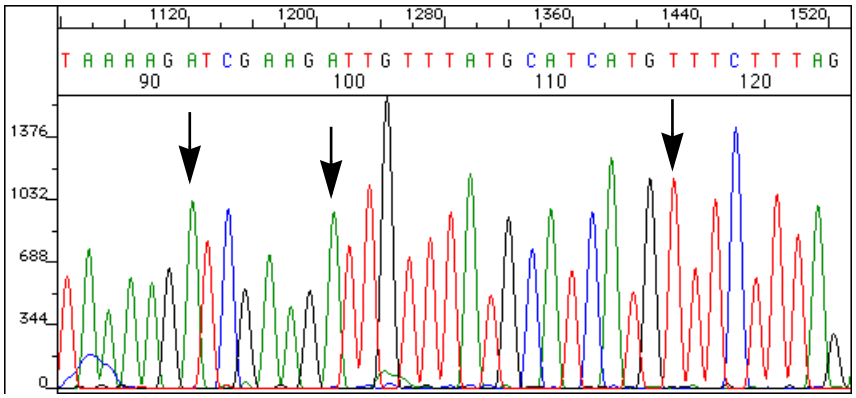


Figure 7-30 Same template as in Figure 7-29, but sequenced with dRhodamine terminators. The arrows point to bases where pull-up peaks were seen with the rhodamine dye terminators

Stop Peaks in PCR Sequencing

Stop Peaks in Dye Primer Chemistry Caused by Primer-Dimer Formation

Sometimes during PCR amplification, the forward and reverse primers form a primer-dimer. If one of the PCR primers is used for sequencing, that primer can anneal to and extend both the PCR fragment and the primer-dimer. In dye primer sequencing, the multiple annealing can create noise in bases up to the end of the primer-dimer and a large stop peak at the end of the primer-dimer (Figure 7-31). This kind of artifact is typically seen in the first 25–60 bases of the sequence, but can extend as far as 100 bases.

The sequence data after the primer-dimer stop peak is often unaffected unless very significant amounts of primer-dimer are present in the reaction. In this case, much of the sequencing primer is used in priming primer-dimer products rather than template molecules and short reads are observed.

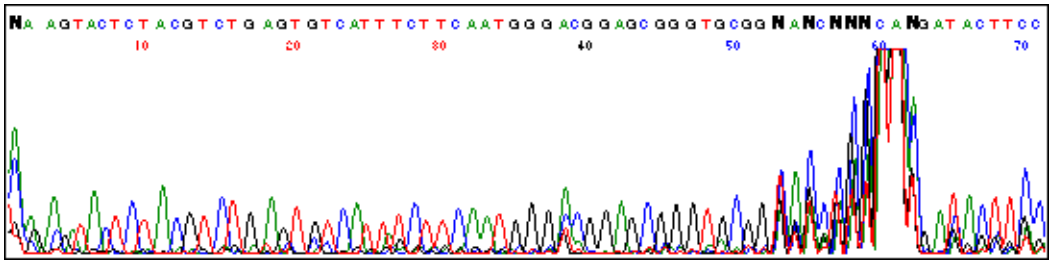


Figure 7-31 Stop peak in dye primer chemistry caused by primer-dimer formation

Stop Peaks in Dye Primer Chemistry Caused by Default Fragments

Sometimes a default fragment is generated from free vector included in the PCR along with the cloned target (vector with insert). During PCR, the vector's multiple cloning region is amplified as well as the intended insert. The resulting default fragment is extended by the dye primers during sequencing, creating a large stop peak the size of the multiple cloning region.

Eliminating Stop Peaks

- Use careful design for your PCR primers to avoid stop peaks caused by primer-dimer formation:
  - Make sure there is no sequence complementarity between the two PCR primers, especially at the 3´ ends.
  - Use a sequencing primer that is different from either of the PCR primers.
  - Ensure that your sequencing primer does not overlap the sequence of the PCR primers.
  - Use a Hot Start technique for the PCR amplification used to generate the sequencing template, *e.g.*, AmpliTaq Gold® DNA Polymerase.
- You can also use dye terminator chemistries to eliminate stop peaks caused by primer-dimers or default fragments.

  With dye terminators, extension products only appear in the sequencing data when a dye-labeled ddNTP is incorporated. Stops are invisible because they are not labeled.

Salt Contamination

Salts used in template preparation can decrease signal strength and read length if not completely removed before sequencing.

Figure 7-32 shows the effect of adding increasing concentrations of sodium chloride (NaCl) to BigDye terminator sequencing reactions before cycle sequencing. Lane 1 has no added salt. Lanes 2–11 have added salt in 10-mM increments from 10–100 mM. At 40 mM NaCl (lane 5), the reduction in read length is apparent.

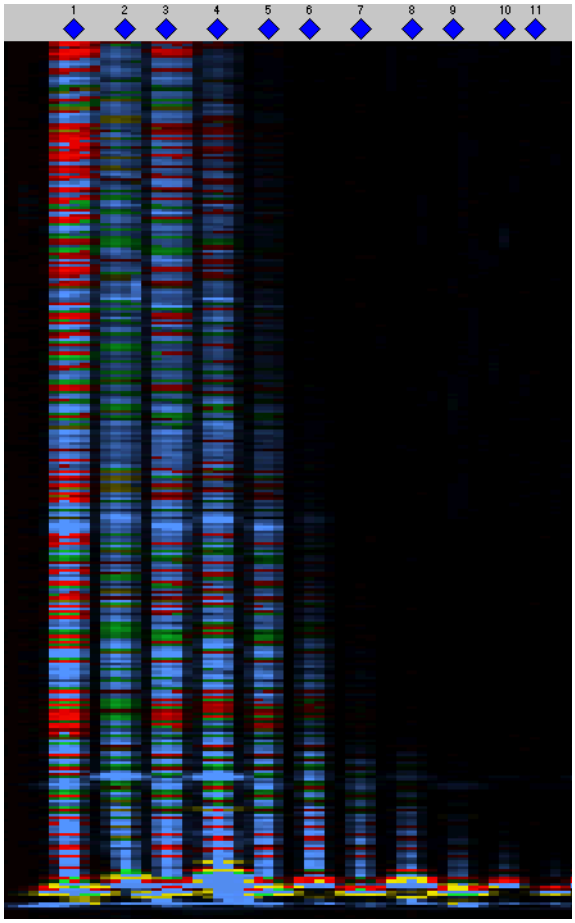


Figure 7-32 Effect of contaminating NaCl on sequencing data

Figure 7-33 on page 7-26 shows the effect of EDTA on BigDye terminator cycle sequencing reactions. The impact on read length is not as great as that of NaCl (Figure 7-32), but there is a steady decrease in signal as EDTA concentration increases (right to left on gel image).

Lane	[EDTA] (mM)
14	1
15	0.8
16	0.6
17	0.4
18	0.3
19	0.2
20	0.1

At EDTA concentrations of 1 mM or higher (up to 6 mM was examined—data not shown), no signal is obtained.

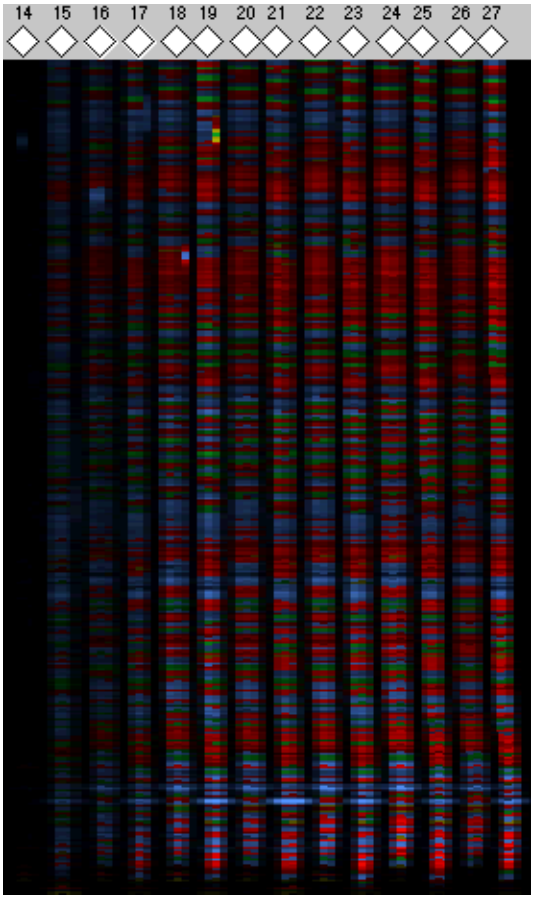


Figure 7-33 Effect of contaminating EDTA on BigDye terminator sequencing data

Salt is not seen in the usual template quality determination methods, such as agarose gel electrophoresis and spectrophotometry. If your data has a short read length, odd peak shape, and low signal strength, the template may be contaminated by salt. See “Cleaning Up Dirty Templates” on page 3-16 for information about removing salt from templates.

Excess Dye Peaks

A common issue occurring with dye terminator cycle sequencing chemistries stems from incomplete removal of unincorporated, fluorescently labeled ddNTPs during alcohol precipitation. In the worst case, residual unincorporated dye can obscure the entire sequence in the first 40 bases (Figure 7-34) and cause scaling problems throughout the sequence (Figure 7-68 on page 7-65).

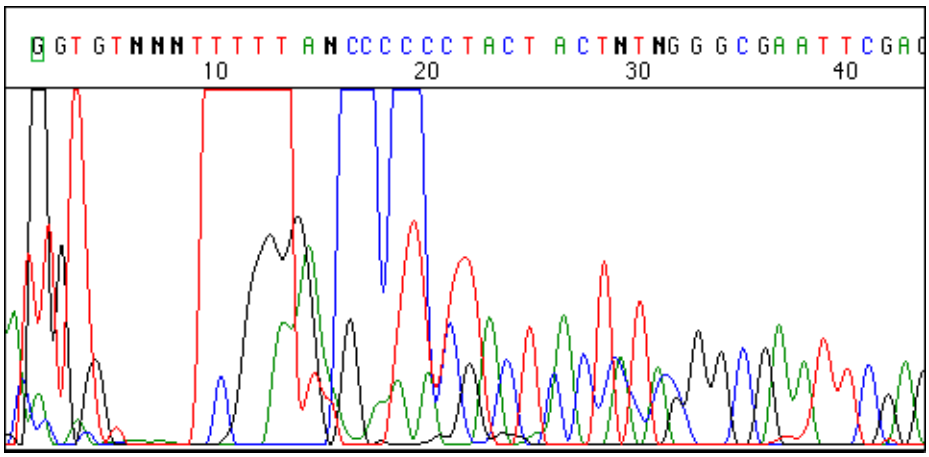


Figure 7-34 Unincorporated dye terminator peaks from a BigDye terminator sequencing reaction

There are several alcohol precipitation methods available for each chemistry (see page 3-33). This is to provide as much flexibility as possible. Use the method that gives the best results in your laboratory.

To avoid excess dye peaks:

- Use only room-temperature alcohol.

  Cold alcohol will also precipitate unincorporated dye terminators.

- Do not use denatured alcohol.

  Denatured alcohol has inconsistent quality. The concentration of the alcohol and purity of the additives can vary.

- Use the correct concentration of alcohol recommended for the method you have chosen as described in “Removing Unincorporated Dye Terminators” on page 3-34.

  Figure 7-35 on page 7-28 shows the effect of ethanol concentration on the precipitation of BigDye terminator sequencing reactions. A 60% concentration removes most of the unincorporated dye terminators without decreasing signal strength. Similar results are obtained with isopropanol.

- Use an appropriate precipitation method for your sequencing chemistry.

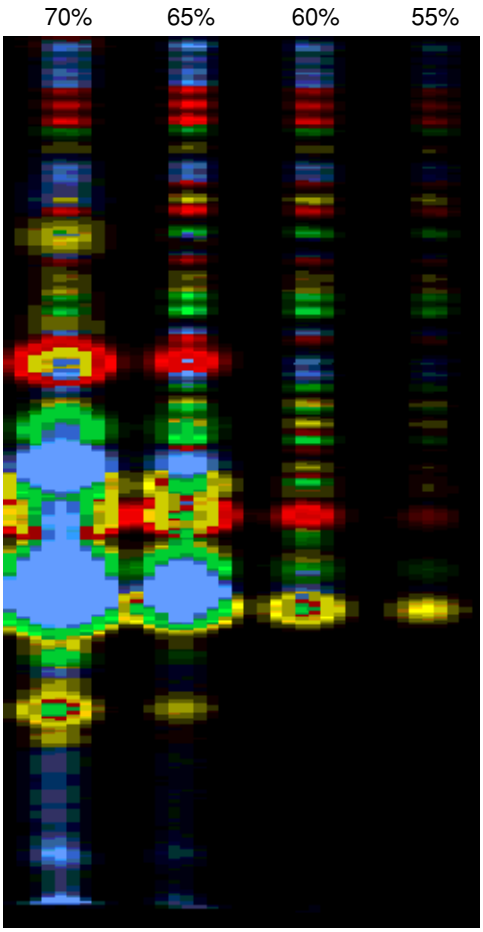


Figure 7-35 Effect of ethanol concentration on BigDye terminator sequencing reaction precipitation

Second- or Third-Panel T Terminator Peak

When removing excess dye terminators from rhodamine dye terminator or BigDye terminator reactions, a broad red peak sometimes appears in the second or third panel of analyzed data (Figure 7-36 on page 7-29).

Often this peak is the result of poor spin column purification procedure. To avoid this problem, load the sample in the center of the column bed. Make sure that the sample does not touch the sides of the column. See page 3-34 for more information on spin column purification.

This peak can also result from poor ethanol precipitation procedures if all of the supernatant is not aspirated after the first centrifugation.

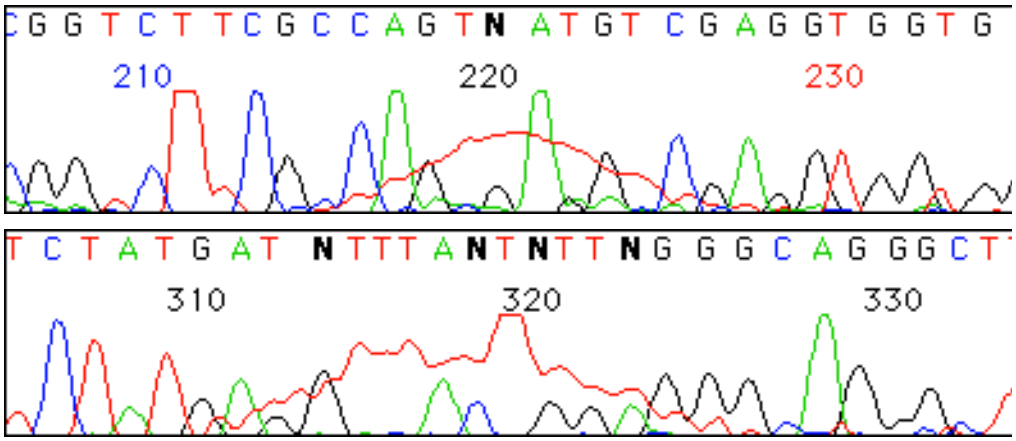


Figure 7-36 The second- or third-panel T peak in rhodamine dye terminator chemistry with AmpliTaq® DNA Polymerase. Two examples are shown.

Troubleshooting DNA Sequence Composition Problems

Overview

DNA sequence compositions cause different problems depending on the sequencing chemistry used. No single chemistry works with all sequences. Modification of a particular method or use of an alternative chemistry may be necessary. This section describes some sequence contexts that cause problems and some of the common sequence-related problems:

- False stops in dye primer chemistry
- Compressions
- GC-rich (>70%) regions of sequence
- Overall GC-rich sequences
- Regions of pronounced secondary structure
- GT-rich regions in BigDye terminator chemistry
- Homopolymer regions
- Repetitive DNA

False Stops in Dye Primer Chemistry

One of the advantages of cycle sequencing is that the high extension temperature discourages the formation of template secondary structures. Certain templates, particularly GC-rich sequences, can still form intrastrand complexes through which AmpliTaq® DNA Polymerase, FS has difficulty extending.

In dye primer sequencing, when the DNA polymerase dissociates from a partially extended fragment without incorporating a dideoxynucleotide terminator, a false stop is seen. If this occurs in all four dye primer reactions, a peak appears in the electropherogram at that position in all four colors (Figure 7-37). In the most severe cases, sequence data stops abruptly.

In dye terminator sequencing, extension products are labeled only if a dye-labeled dideoxynucleotide is incorporated. If the enzyme falls off the template at a region of secondary structure and no dye-labeled dideoxynucleotide is incorporated, the fragment is not detected (Figure 7-38 on page 7-31).

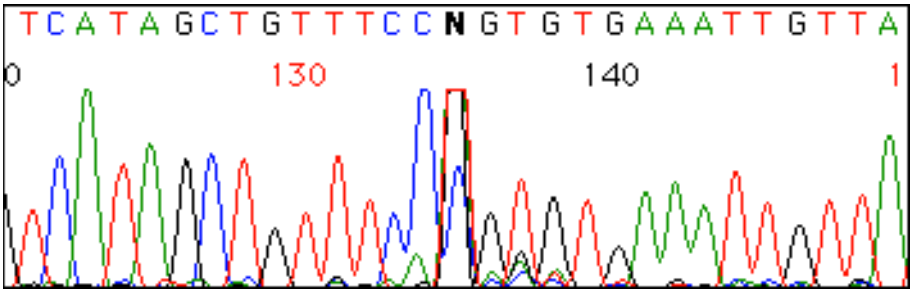


Figure 7-37 A false stop in pGEM control DNA sequenced using fluorescein/rhodamine dye primer chemistry. A peak is seen in all four colors at the position of the N on the electropherogram where secondary structure creates a false stop for the polymerase.

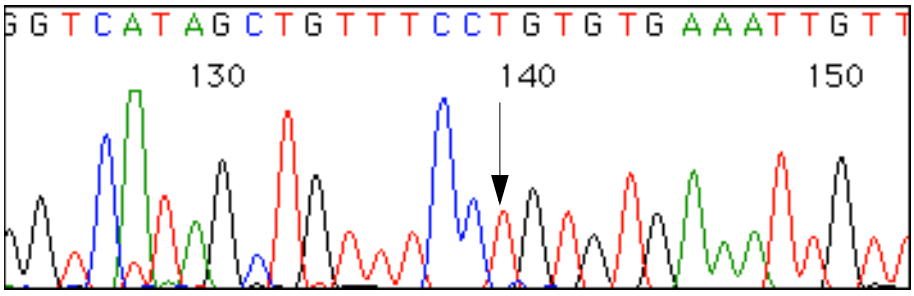


Figure 7-38 The same region of pGEM control DNA sequenced with dye terminators. The site of the false stop evident in Figure 7-37 on page 7-30 is not seen when using dye terminators. An arrow marks the base that was called as an N in Figure 7-37.

Ways to Obtain the Sequence

There are two ways to obtain the sequence at false stops when Ns are called:

- Sequence the region using a dye terminator chemistry (Figure 7-38).
- Sequence the opposite strand. False stops rarely occur at exactly the same base position on both strands.

Compressions

Band compressions in DNA sequencing result from the formation of secondary structures in the DNA fragments that are not eliminated by the denaturing conditions of the gel (Mills and Kramer, 1979). The fragments do not migrate according to their size, and more than one fragment can migrate at the same position.

In dye primer chemistries, 7-deaza-dGTP is used to minimize problems with compressions (Barr *et al.*, 1986; Mizusawa *et al.*, 1986), but it is not effective at eliminating all of them (Figure 7-39).

In the fluorescein/rhodamine dye primer sequencing data shown in Figure 7-39, compressions are present at bases 257 and 323 (see arrows).

Note Stop peaks are present at bases 304 and 308.

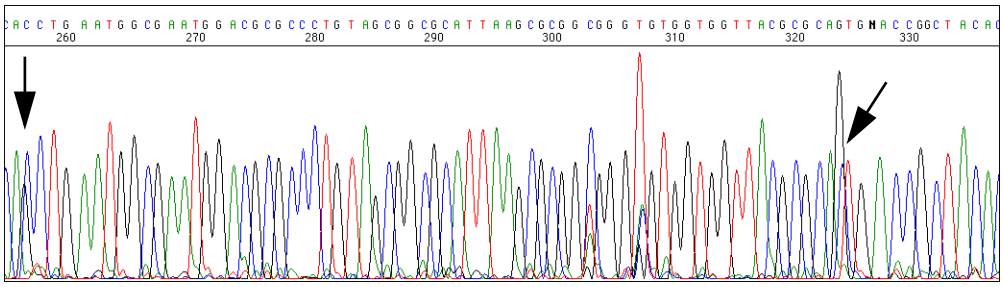


Figure 7-39 Compressions in pGEM control DNA sequenced with the M13 Reverse primer using fluorescein/rhodamine dye primer chemistry on an ABI PRISM 377 DNA Sequencer

In dye terminator reactions, dITP is used in place of dGTP. This eliminates most compressions (Figure 7-40 on page 7-32).

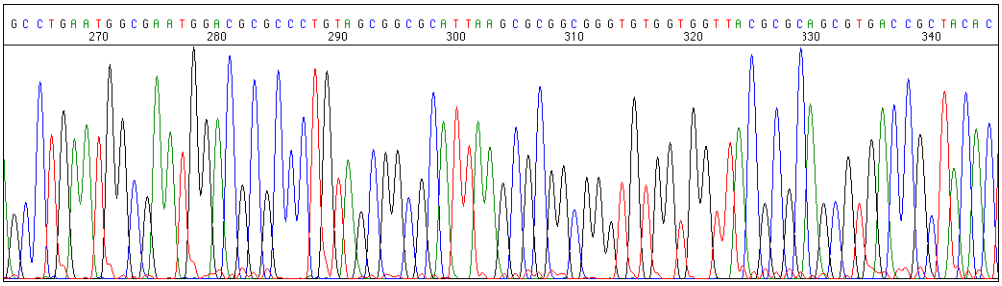


Figure 7-40 Same template as in Figure 7-39 on page 7-31, but sequenced using BigDye terminator chemistry

When problems are encountered with compressions in dye primer data, there are three ways to resolve them:

- Sequence the DNA template using a dye terminator chemistry.
- Sequence the complementary strand, if possible.

  Compressions rarely occur at the same position in both strands of a template.
- Increase the denaturing power of the gel or polymer by using a higher run temperature and/or by increasing the concentration of denaturant. An additional denaturant such as formamide can be used in slab gels.

  These changes can affect the resolution of the gel or polymer and tend to decrease the read length, which makes them less than ideal.

GC-Rich Templates or GC-Rich Regions

Templates with a GC content greater than 70% can be difficult to sequence when using the standard reaction conditions. This is probably related to the higher melting temperature of the DNA caused by the higher proportion of GC base pairs. Even a template that has a fairly average base composition overall can have a very GC-rich region that affects its ability to be sequenced.

The most common problem seen with GC-rich templates is weak signal. Figure 7-41 shows data that was obtained with GC-rich DNA using BigDye terminators and the standard cycling conditions. The signal strengths of the four bases are G: 63, A: 34, T: 26, and C: 64. The increased T noise (red) is due to the software scaling up the low T signal.

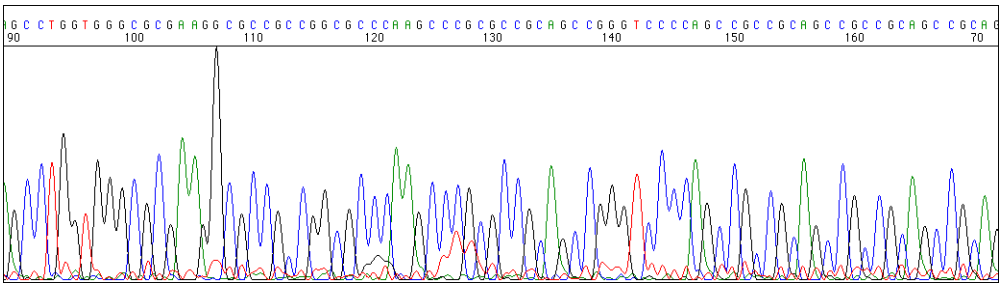


Figure 7-41 GC-rich template sequenced using BigDye terminators under standard conditions

When the denaturation temperature was raised from the standard 96 °C to 98°C, the signals obtained were increased to G: 481, A: 241, T: 181, and C: 498 (Figure 7-42 on page 7-33).

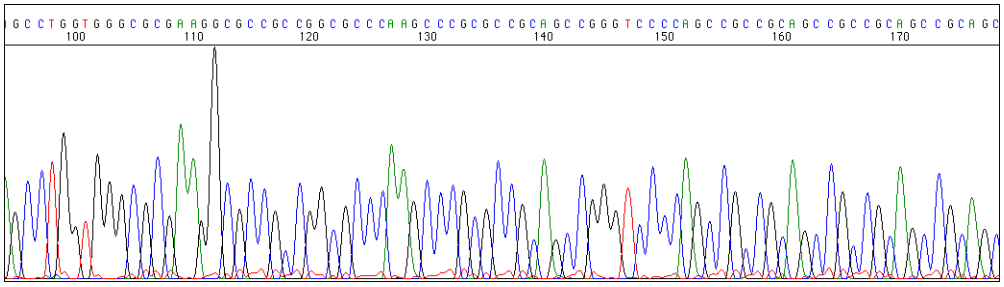


Figure 7-42 GC-rich template sequenced using BigDye terminators with a 98 °C denaturation temperature

Suggested Approaches for GC-Rich Templates

- Increase the denaturation temperature to 98 °C.
- Add DMSO to a final concentration (v/v) of 5% (Burgett *et al.*, 1994; Landre *et al.*, 1995).

  Addition of a mixture of 5% DMSO and 5% glycerol has also been used successfully for some templates.
- Incubate the reaction at 96 °C for 10 minutes before cycling.
- Add betaine to a final concentration of 1M (Henke *et al.*, 1997; Baskaran *et al.*, 1996).
- Double all reaction components and incubate at 98 °C for 10 minutes before cycling.
- Add 5–10% formamide or 5–10% glycerol to the reactions.
- Linearize plasmids with a restriction enzyme.
- Shear the insert into smaller fragments (<200 bp) and subclone.
- Amplify the DNA with substitution of 7-deaza-dGTP for 75% of the dGTP in the PCR, then sequence the PCR product (Innis, 1990; Fernandez-Rachubinski *et al.*, 1990).

Secondary Structure in the Template

The presence of secondary structure in the template strand often results in difficulty obtaining good sequence data beyond the region of secondary structure. Many of the same approaches that are useful for sequencing GC-rich templates are also useful for templates that have regions of strong secondary structure. An approach that sometimes works is to use a primer that anneals close to the region of signal loss. The use of short-insert libraries has also been used to solve problems of secondary structure in a genome sequencing project (McMurray *et al.*, 1998).

Sequence of a GT-Rich Template Obtained with BigDye Terminators

Figure 7-43 shows a portion of sequence data obtained with BigDye terminators. The insert in this clone is from *Dityostelium japonicum*. The base composition is approximately 30% GC. However, the distribution of bases is significantly different in the two strands.

In the strand from which the data shown here was obtained, there is a higher proportion of Cs and As, which results in a high proportion of Gs and Ts in the synthesized strand. In particular in the region of bases 192–250, 52 of 58 bases are either G or T, *i.e.*, 90%. After this region the sequence data obtained with BigDye terminator chemistry dies (Figure 7-43).

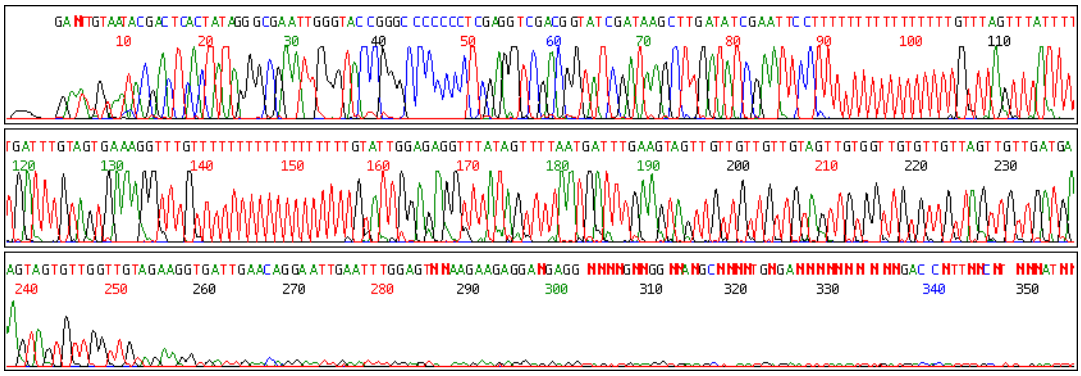


Figure 7-43 A template with a very GT-rich region sequenced using BigDye terminator chemistry. Note the lack of signal after the GT-rich region.

When the same template was sequenced with dRhodamine terminators, good data was obtained beyond the GT-rich region (Figure 7-44).

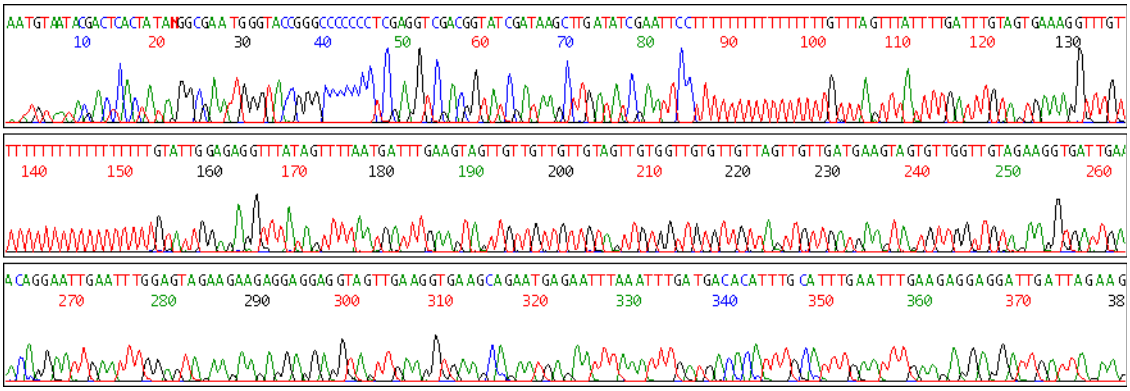


Figure 7-44 Same template as in Figure 7-43, but sequenced with dRhodamine terminators

In both chemistries dITP is used in place of dGTP, but in the BigDye terminator chemistry the dTTP has been replaced with dUTP as well. Both dITP and dUTP will lower the melting temperature of DNA.

Presumably with such a high concentration of Gs and Ts in this region, the duplex formed by the extended primer and the template is less stable. The polymerase has difficulty extending through the region.

Further studies with this template showed that replacement of either the dITP with dGTP or the dUTP with dTTP allowed good extension through this region (data not shown).

The following methods were found useful at Applied Biosystems to improve the sequencing results for this template:

- Lowering the extension temperature from 60°C to 55°C or 50°C

Note Lowering the extension temperature will result in some loss of signal strength.

- Addition of 1 mM magnesium chloride to the reaction, which increases the final concentration in the BigDye terminator reaction from 2 mM to 3 mM magnesium chloride.
- Using dRhodamine terminator chemistry (Figure 7-44 on page 7-34)

At the present time the exact composition of a GT-rich region that is sufficient to cause this problem with BigDye Terminators is unknown.

Homopolymer Regions

With Cloned DNA

Long homopolymer T regions (or A regions) can cause problems in DNA sequencing reactions due to “slippage” in the region of the homopolymer. Although the sequence data can be clean through the homopolymer region, the data after this region is noisy due to the presence of multiple sequences (Figure 7-45).

The exact mechanism of slippage is not known. Presumably, the two strands do not stay paired correctly during polymerization through the homopolymer region. This generates fragments with homopolymer regions of different length that have the same sequence after that region.

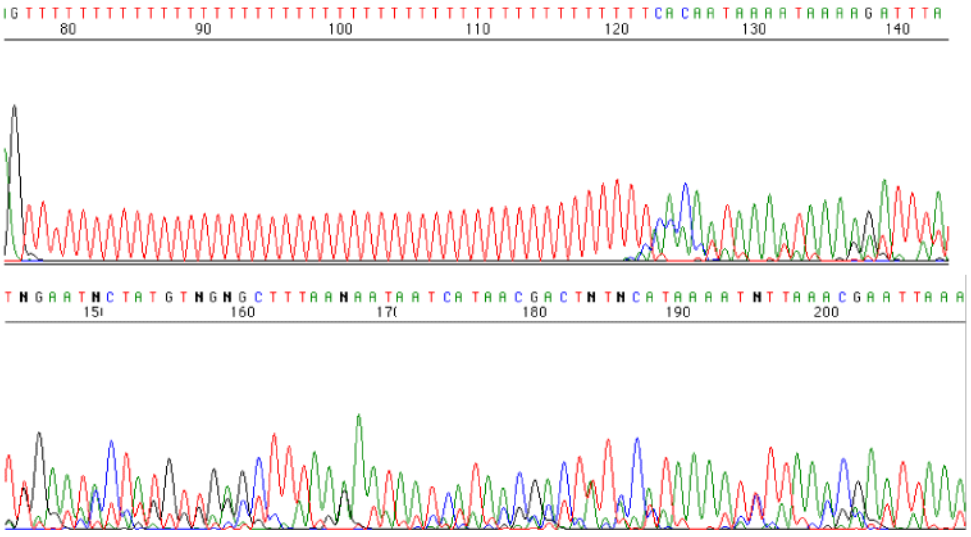


Figure 7-45 Homopolymer region sequenced using BigDye terminator chemistry

The occurrence of slippage is length dependent and short homopolymer regions are rarely problematic in sequencing reactions. Slippage is more of a problem with T regions (A in the template strand) in BigDye terminator reactions due to the use of dUTP in the deoxynucleotide mixture.

When the same template shown in Figure 7-45 is sequenced with dRhodamine terminators, the sequence data is much cleaner (Figure 7-46).

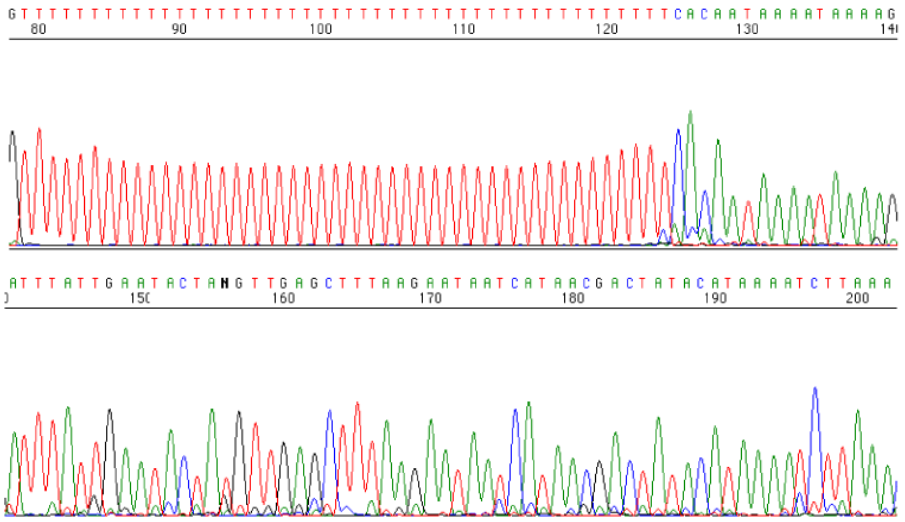


Figure 7-46 Homopolymer A region sequenced using dRhodamine terminator chemistry

Good sequence data immediately past a polyA region can be obtained by using an anchored primer, a sequencing primer that is polyT containing an A, C, or G base at its 3´ end (Figure 7-47). The 3´ base anchors the primer into place at the end of the homopolymer region (Khan *et al.*, 1991; Thweatt *et al.*, 1990; Thomas *et al.*, 1993).

Note When sequencing with a polyT primer that has a "wobble" (A, C, or G at the 3´ end), use 3.2 pmol of each primer, *i.e.*, 9.6 pmol total of primer.

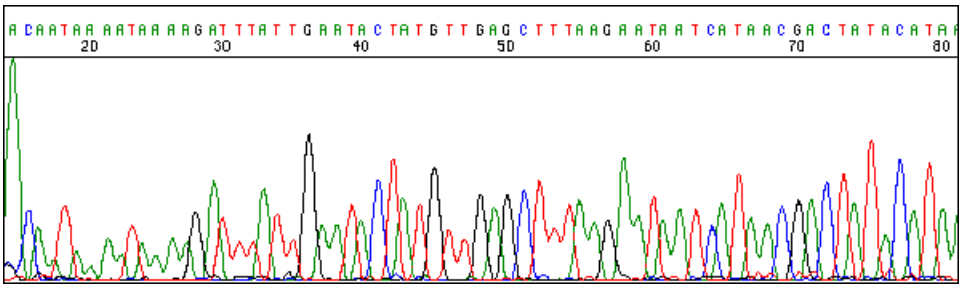


Figure 7-47 Sequencing past a polyA region in the template using a primer with the sequence $T_{25}G$. The sequence past the homopolymer region is improved markedly over that shown in Figure 7-45 on page 7-35).

With PCR Templates

Slippage also occurs in PCR amplification and is a common problem when sequencing PCR products. Slippage can occur at regions that normally are not problematic to sequence with cloned DNA.

Sequence data is shown below from a plasmid clone in which the sequencing template was prepared by amplification of the insert (Figure 7-48 on page 7-37) or by isolation of the plasmid DNA (Figure 7-49 on page 7-37). After the homopolymer G region, the sequence data is unusable for the amplified template. When the plasmid

DNA was sequenced directly, the sequence data was weak but unambiguous after the homopolymer G stretch.

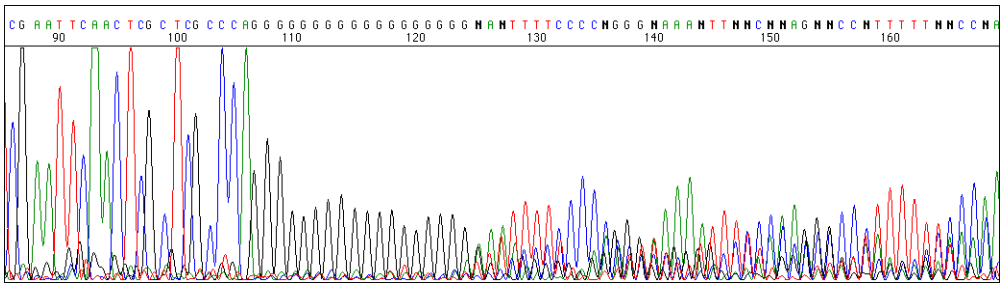


Figure 7-48 Fluorescein/rhodamine dye primers with AmpliTaq® DNA Polymerase, CS+ were used to sequence a template obtained from a plasmid clone by PCR amplification of the insert

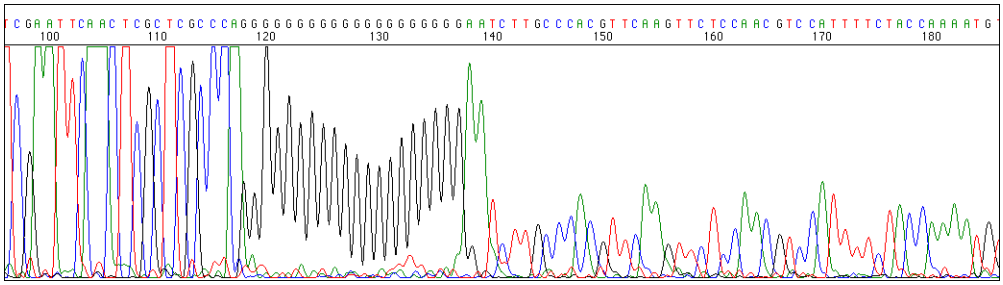


Figure 7-49 BigDye terminator data from the same plasmid clone as in Figure 7-48, but the template was obtained by isolation of the plasmid DNA

At the present time there is no easy solution for the problem of slippage in PCR amplifications. There are three approaches that can be used to obtain the sequence data after such a region in PCR-generated templates:

- Anchored primers can sometimes be used to obtain sequence data after homopolymer T regions (see page 7-36).
- Sequence the complementary strand.

  This will give good sequence for the ambiguous region up to the homopolymer region, but the same problem will occur afterwards. It can be difficult to determine the exact number of bases present in the homopolymer region.
- Clone the PCR product.

  In many cases the cloned product will not show any evidence of slippage when sequenced. Multiple clones need to be sequenced to be certain that the correct species has been identified. The main disadvantage of this is the work required to isolate and sequence a reasonable number of clones.

Repetitive DNA

DNA containing short repetitive regions (up to 200-300 bases) is generally not difficult to sequence unless the base composition of the repeat is problematic. For example, with a relatively short CGG repeat consisting of 38 repeat units, we have had difficulty getting good sequence data beyond the repeat. In this case, the DNA was handled as for GC rich templates.

When the length of the repeat is more than 500 bases it can be difficult to get good sequence data from both strands. Since there is generally no unique sequence in these repeats, synthesis of walking primers is not an option. Two approaches have been used successfully with such clones:

- Use of directed deletions
- Use of an *in vitro* transposon system, such as the Primer Island Transposition Kit (Devine and Boeke, 1994; Devine *et al.*, 1997)

  Refer to the *Primer Island Transposition Kit Protocol* (P/N 402920) for more information about this kit.

Troubleshooting Sequencing Data

Troubleshooting Sequencing Data

Observation	Possible Causes	Recommended Actions
No recognizable sequence (see page 7-10)	Insufficient template	Quantitate the DNA template. Increase the amount of DNA in the sequencing reactions. See page 3-17.
	Inhibitory contaminant in template	Clean up the template. See page 3-16.
	Insufficient primer	Quantitate the primer. Increase the amount of primer in the sequencing reactions. See page 3-19.
	Primer has no annealing site	Use a primer that is complementary to the template.
	Poor primer design or incorrect primer sequence	Redesign the primer. See page 3-18.
	Missing reagent	Repeat reactions following the protocol carefully. See page 3-21.
	Old or mishandled reagents	Use fresh reagents. See page 3-20.
	Thermal cycler power failure	Repeat reactions.
	Thermal cycling conditions	Calibrate the thermal cycler regularly.
		Use the correct thermal cycling parameters.
		Use the correct tube for your thermal cycler.
		Set ramp rates to 1 °C/second.
	Extension products lost during reaction cleanup	Ensure that correct centrifugation speeds and times are used for precipitation and spin column procedures. See page 3-33
	Extension products not resuspended	Resuspend sample pellet in loading buffer or TSR carefully.
	Lane tracking failure (ABI 373 or ABI PRISM 377 DNA Sequencer)	Check lane tracking. Retrack and reextract lanes if necessary.
	Electrokinetic injection failure (ABI PRISM 310 Genetic Analyzer)	Repeat injections.

Troubleshooting Sequencing Data *(continued)*

Observation	Possible Causes	Recommended Actions
Noisy data throughout sequence, with low signal strength (see page 7-11)	Not enough DNA in the sequencing reactions	Use more DNA in the sequencing reactions.
		Load or inject more of the resuspended sequencing reactions. See “Preparing and Loading Samples for Gel Electrophoresis” on page 3-50 or “Preparing and Loading Samples for Capillary Electrophoresis” on page 3-53.
	GC-rich template or GC-rich region in template	Increase the denaturation temperature to 98 °C.
		Add DMSO to a final concentration (v/v) of 5%.
		Incubate the reaction at 96 °C for 10 minutes before cycling.
		Double all reaction components and incubate at 98 °C for 10 minutes before cycling.
		Add 5–10% glycerol or 5–10% formamide to the reactions.
		Linearize the DNA with a restriction enzyme.
		Shear the insert into smaller fragments (<200 bp) and subclone.
		Amplify the DNA using 7-deaza-dGTP in the PCR, then sequence the PCR product.
	Expired or mishandled reagents	Use fresh reagents. See page 3-20.
	Thermal cycling conditions	Calibrate the thermal cycler regularly.
		Use the correct thermal cycling parameters.
		Use the correct tube for your thermal cycler.
		Set ramp rates to 1 °C/second.
	Lane tracking failure	Check lane tracking. Retrack and reextract lanes if necessary.
Noisy data throughout sequence, with good signal strength (see page 7-11	Contaminated template	Clean up the template. See page 3-16.
	Multiple templates in sequencing reaction	Examine your template on an agarose gel to see that only one template is present. See page 3-16.
	Multiple priming sites	Ensure that your primer has only one priming site. Redesign the primer if necessary. See page 3-18.
	Multiple primers when sequencing PCR products	Purify your PCR template to remove excess primers. See page 3-12.
	Primer with N–1 contamination	Use HPLC-purified primers.
	High signal saturating detector	Use less DNA in the sequencing reactions or load less on the gel or into the capillary.
	Incorrect run module	Use the correct run module. See page 6-2.
	Incorrect instrument (matrix) file	Use the correct instrument file for your sequencing chemistry. See page 6-7 for information on creating instrument files.

Troubleshooting Sequencing Data *(continued)*

Observation	Possible Causes	Recommended Actions
Noise up to or after a specific point in the sequence (see page 7-12)	Mixed plasmid preparation	Ensure that you have only one template. See "Plasmid DNA Templates" on page 3-6 and "Determining DNA Quality" on page 3-16.
	Multiple PCR products	Ensure that you have only one template. See "Preparing PCR Products for Sequencing" on page 3-12 and "Determining DNA Quality" on page 3-16.
	Primer-dimer contamination in PCR sequencing	Optimize your PCR amplification. See page 3-10.
		Make sure there is no sequence complementarity between the two PCR primers.
		Use a sequencing primer that is different from either of the PCR primers.
		Ensure that your sequencing primer does not overlap the sequence of the PCR primers.
		Use a Hot Start technique, *e.g.*, AmpliTaq Gold DNA Polymerase.
	Slippage after repeat region in template	Try an alternate sequencing chemistry. See page 2-15.
		Use an anchored primer. See page 7-36.
Poor mobility correction (see page 7-13)	Incorrect dye set/primer (mobility) file	Use the correct mobility file. See page 6-5.
	Incorrect Peak 1 Location for data analysis	Choose a new Peak 1 Location. See page 6-15.
	Gel with very different separation properties from the gel matrices that were used to construct the dye set/primer (mobility) files	Use the correct dye set/primer file for your gel type. Refer to the *ABI PRISM DNA Sequencing Analysis Software User's Manual*.
Early signal loss (see page 7-14)	Region of secondary structure in the template	Sequence the opposite strand.
		Use a sequencing primer that anneals at a different position.
		Try an alternate sequencing chemistry. See page 2-15.
		Incubate the reaction at 96 °C for 10 minutes before cycling.
		Increase the denaturation temperature to 98 °C.
		Increase the extension temperature by 2–3 °C.
	GT-rich regions with BigDye terminators (see page 7-34)	Decrease the extension temperature in cycle sequencing to 55 °C or 50 °C.
		Increase the magnesium ion concentration by 1 mM.
		Sequence the opposite strand.
		Try an alternate sequencing chemistry. See page 2-15.

Troubleshooting Sequencing Data *(continued)*

Observation	Possible Causes	Recommended Actions
Early signal loss (see page 7-14)	GC-rich region in template	Increase the denaturation temperature to 98 °C.
		Add DMSO to a final concentration (v/v) of 5%.
		Incubate the reaction at 96 °C for 10 minutes before cycling.
		Double all reaction components and incubate at 98 °C for 10 minutes before cycling.
		Add 5–10% glycerol or 5–10% formamide to the reactions.
		Linearize the DNA with a restriction enzyme.
		Shear the insert into smaller fragments (<200 bp) and subclone.
		Amplify the DNA using 7-deaza-dGTP in the PCR, then sequence the PCR product.
	Poor lane tracking, such that tracker line diverges from the data	Check lane tracking. Retrack and reextract lanes if necessary.
	Poor quantitation of primer	Quantitate the primer. See page 3-19.
	Poor quantitation of template	Quantitate the DNA template, especially with PCR products. See page 3-17.
Excess dye peaks at the beginning of the sequence in dye terminator chemistries (see page 7-27)	Poor removal of unincorporated dye terminators	Choose the Start Point for data analysis to exclude the excess dye peaks. See page 6-18.
		Follow the protocols for excess dye terminator removal carefully. See page 3-33. Refer also to the *Precipitation Methods to Remove Residual Dye Terminators from Sequencing Reactions User Bulletin* (P/N 4304655). This document can be obtained from the Applied Biosystems WWW site (www.appliedbiosystems.com/techsupport).
		When using Centri-Sep spin columns, be careful to load the sample onto the center of the gel surface. Do not touch the gel surface with the pipet tip. See page 3-34. **IMPORTANT** When using BigDye terminators, be sure to hydrate the column for at least 2 hours.
		Spin samples in the centrifuge for the recommended times. Spinning too long precipitates more dyes with the sample.
		When working with microcentrifuge tubes, aspirate the supernatant rather than decant it. Decanting leaves excess ethanol on the sides of the tube.

Troubleshooting Sequencing Data *(continued)*

Observation	Possible Causes	Recommended Actions
Broad, red peak between base 200 and 350 (see page 7-28)	Poor removal of unincorporated dye terminators	Follow the protocols for excess dye terminator removal carefully. See page 3-33. Refer also to the *Precipitation Methods to Remove Residual Dye Terminators from Sequencing Reactions User Bulletin* (P/N 4304655). It can be obtained from the Applied Biosystems WWW site (www.appliedbiosystems.com/techsupport).
		When using Centri-Sep spin columns, be careful to load the sample onto the center of the gel surface. Do not touch the gel surface with the pipet tip. See page 3-34. **IMPORTANT** When using BigDye terminators, be sure to hydrate the column for at least 2 hours.
Pull-up peaks/ bleedthrough (see page 7-22)	Total signal strength above 4000	Quantitate the DNA template (see page 3-17). Use less template.
		Load or inject less of the resuspended sequencing reactions. See “Preparing and Loading Samples for Gel Electrophoresis” on page 3-50 or “Preparing and Loading Samples for Capillary Electrophoresis” on page 3-53.
Stop peaks in dye primer chemistry	Primer-dimer contamination in PCR sequencing (see page 7-24)	Optimize your PCR amplification. See page 3-10.
		Make sure there is no sequence complementarity between the two PCR primers, especially at the 3´ end.
		Use a Hot Start technique for the PCR amplification used to generate the sequencing template, *e.g.*, AmpliTaq Gold DNA Polymerase.
		Use a dye terminator sequencing chemistry.
	Default fragments in PCR sequencing of plasmid inserts (see page 7-24)	Ensure that you have only one template. See “Plasmid DNA Templates” on page 3-6 and “Determining DNA Quality” on page 3-16.
	DNA sequence composition (see page 7-30)	Use a dye terminator sequencing chemistry. See page 2-2.
		Sequence the opposite strand.
Compressions (see page 7-31)	Sequence-dependent region of anomalous mobility, particularly with dye primer chemistries	If using dye primer chemistry, try a dye terminator sequencing chemistry. See page 2-2.
		Sequence the opposite strand.
		Increase the denaturing ability of the gel or polymer by using higher run temperatures or denaturing agents such as formamide. **Note** This can decrease the resolution of the gel or polymer and give shorter read lengths.
Poor data following a long homopolymer region (see page 7-35)	Slippage	Try an alternate sequencing chemistry.
		Use an anchored primer to determine sequence after a homopolymer T region in the sequence (A region in the template strand). See page 7-36.

Troubleshooting Gel Electrophoresis on the ABI 373 and ABI PRISM 377

Overview

This section shows examples of common problems that can occur with gel electrophoresis. Refer to the table on page 7-53 for a more complete guide to troubleshooting gel electrophoresis.

Poor-Quality Acrylamide

Use fresh, high-quality acrylamide. Poor quality acrylamide contains acrylic acid (a deamidation product) and linear polyacrylamide, which will copolymerize and cause local pH changes in the gel. This causes streaking and smearing of bands (Figure 7-50).

During storage at room temperature, especially in water, acrylamide breaks down into acrylic acid. Prepare only as much acrylamide-bisacrylamide solution as you will need in a month.

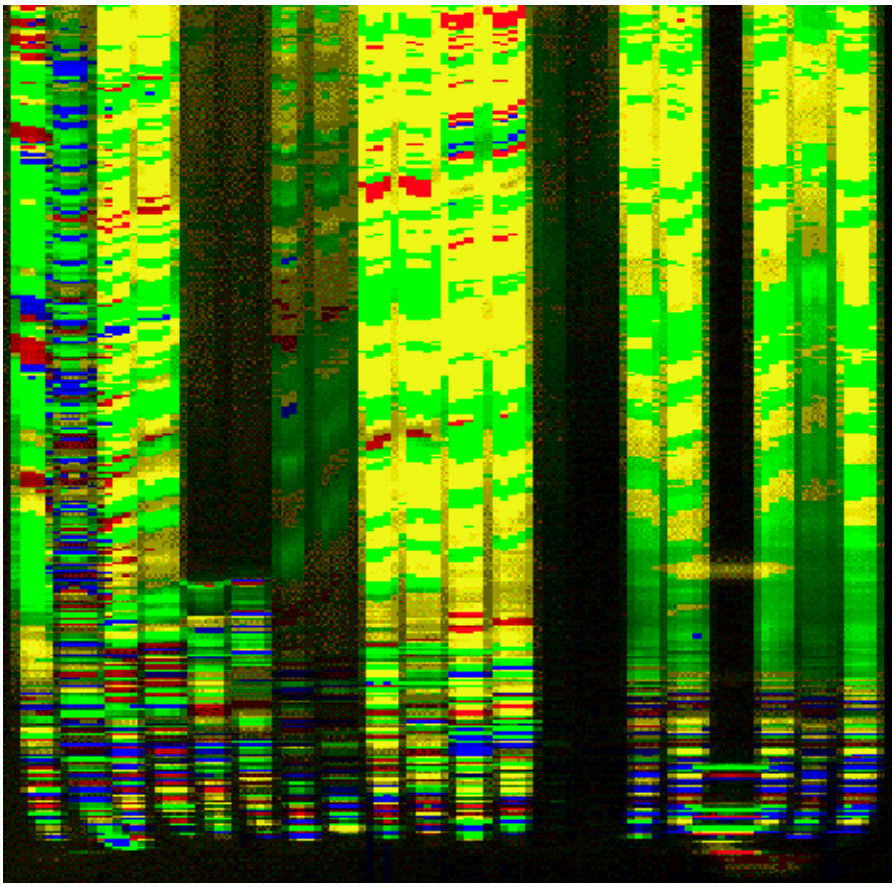


Figure 7-50 Effect of poor quality acrylamide on sequencing data

Salt Excess salt in the wells can cause pinching of lanes toward the center of the gel (Figure 7-51).

Note Lanes 3–8 are short PCR products. Lane 11 was not loaded.

When performing ethanol precipitation, remove all of the ethanol by aspiration after the first spin. If residual ethanol is dried down with the sample, the pinching and bending of lanes is worsened. Performing a 70% ethanol wash after ethanol precipitation of dye terminator reactions also helps to alleviate this problem.

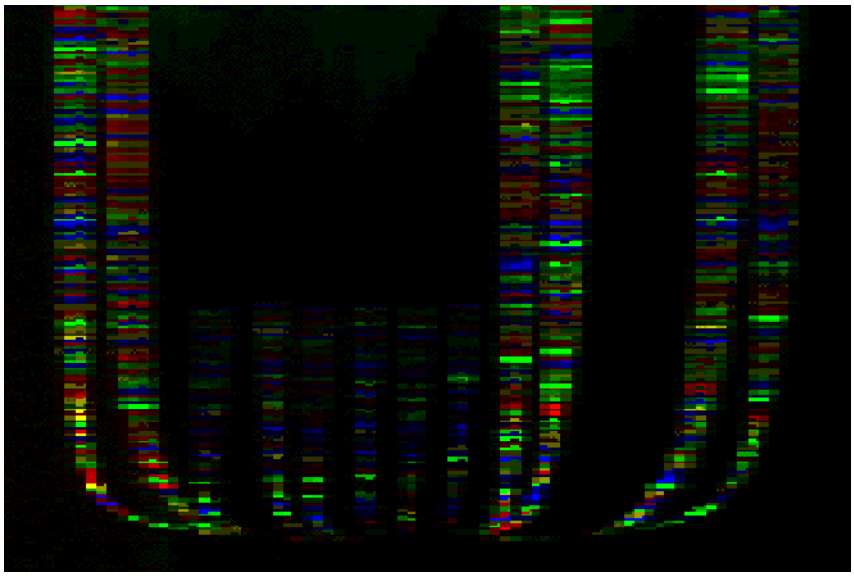


Figure 7-51 Effect of excess salt on an ABI PRISM 377 gel. The overall signal strength is also lowered.

Fluorescent Contaminants

Contaminating fluorescent species can obscure sequencing data completely. A common cause of fluorescent contamination is ink from marker pens. Do not write on the gel plates, spacers, combs, or buffer chambers.

In Figure 7-52, a large green band is seen shortly after the first fragments are detected. The band is from a marking pen that was used to label a spacer. Even though the spacer was cleaned before use, enough ink remained to ruin the sequencing data.

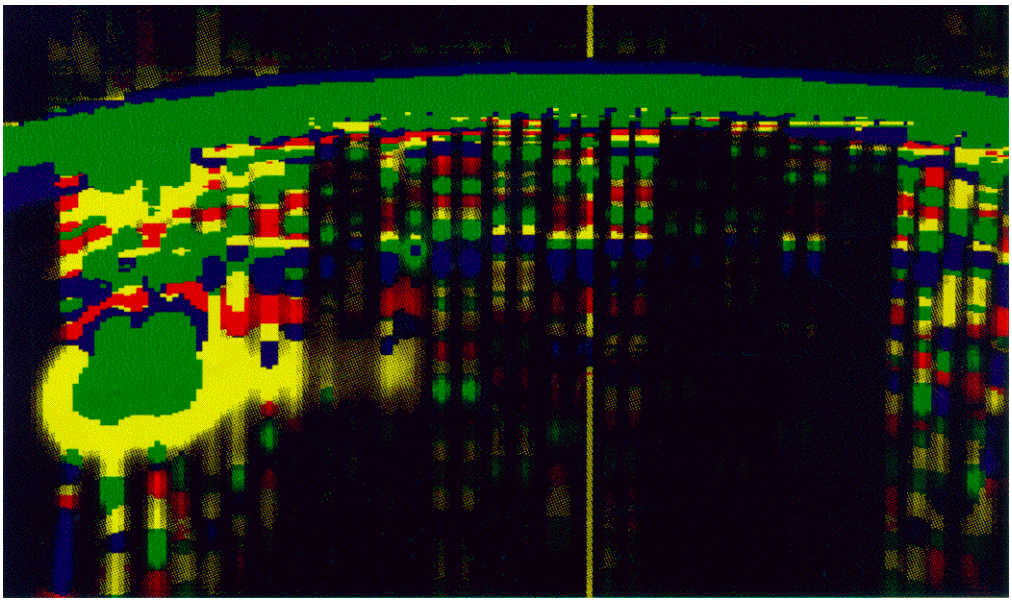


Figure 7-52 Fluorescent contamination from a marking pen

Buffer Leaks

If buffer spills or leaks onto the read region of the gel plates, it can cause blue or green artifacts on the gel image (Figure 7-53).

To avoid buffer leaks, make sure that the plates are clamped correctly and that the upper buffer chamber gasket makes a proper seal. Do not spill buffer behind the upper buffer chamber, as wicking can occur.

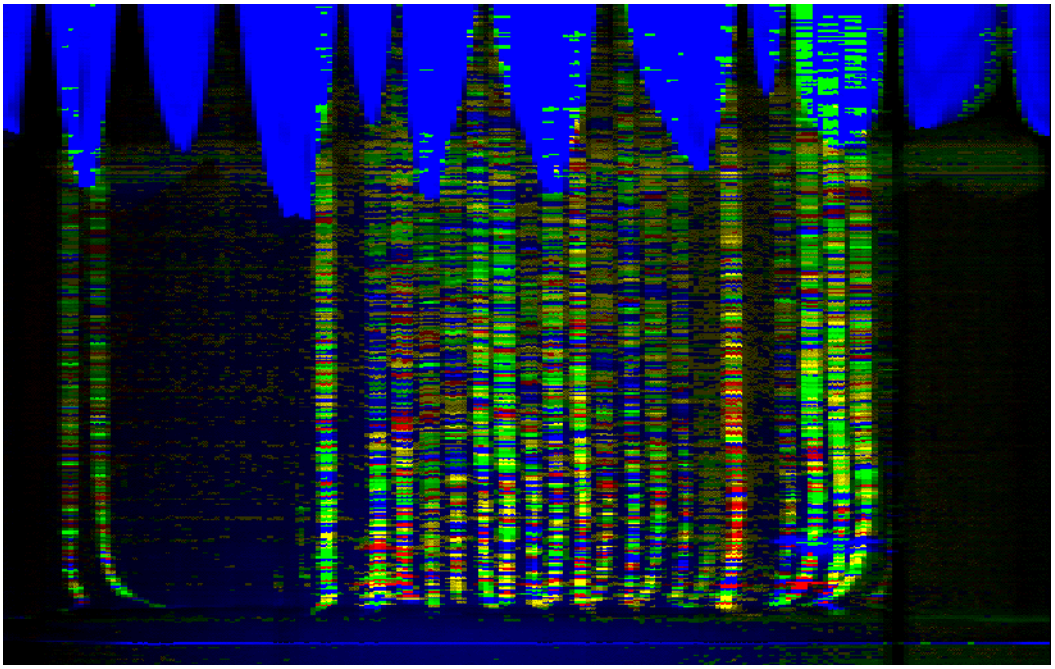


Figure 7-53 Buffer leak in the read region of the plates

Buffer leaks or evaporation also can cause electrophoresis failure if there is not enough buffer for electrophoresis. Note that electrophoresis fails at the same point in each sample (Figure 7-54 on page 7-48), causing diffuse bands to appear throughout the rest of the run.

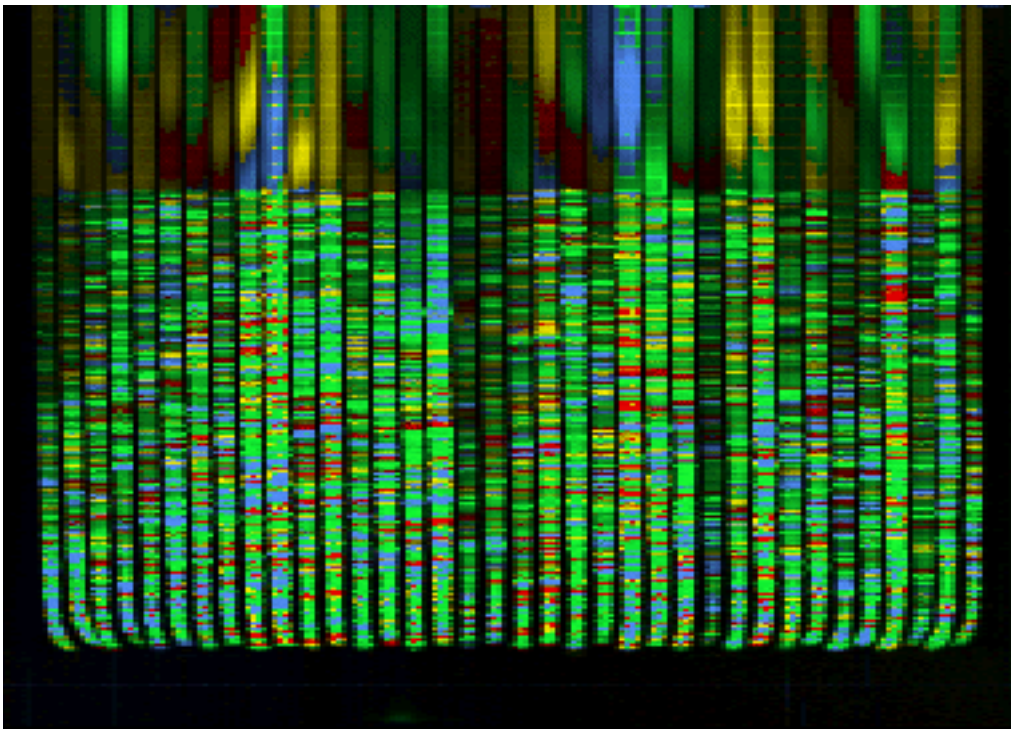


Figure 7-54 Electrophoresis failure caused by a buffer leak

To prevent this from happening:

- Clean the front plate well, so the gasket will make a good seal.
- Use the lid on the upper buffer chamber.
- Take care when filling the upper buffer chamber not to spill buffer behind it.
- Do not fill the upper buffer chamber to the top because buffer will wick over the ears of the notched plate and run down the sides or back of the gel plates.
- Check gasket for leaks before starting the run.

Red Rain

Gel destruction in the read region of the gel can cause red streaks in the data, often near the end of the run (and therefore near the top of the gel image). This effect, shown in Figure 7-55, is known as “red rain.”

Gel destruction often results from drying out of the gel, and is exacerbated by extreme run conditions, *e.g.*, high voltage, high power, high temperature, and long run times.

The following can be used to help prevent red rain:

- Wrap the gel plates to prevent the gel from drying out.
- Lower the run temperature from 51 °C to 48 °C.
  - A lower temperature results in a slower run speed. Less data is collected in the same run time.
  - A lower temperature also means less denaturing power in the gel, which can lead to more extension product secondary structure in the gel. This can result in more compressions, particularly with dye primer chemistries (see page 7-31).

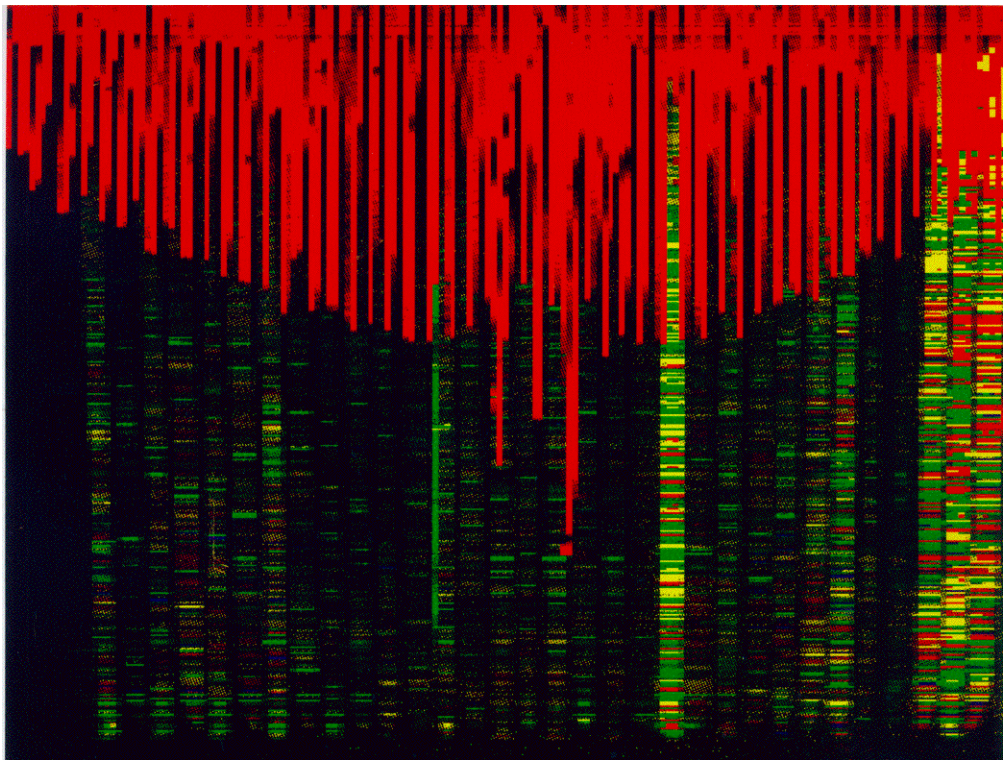


Figure 7-55 “Red rain” on a 48-cm, 36-lane ABI PRISM 377 gel

Gel Extrusion

When voltage is applied on the ABI PRISM 377 DNA Sequencer, the polyacrylamide gel sometimes moves from between the glass gel plates toward the cathode (upper electrode) and into the upper buffer chamber. In extreme cases, up to about five centimeters of gel in a folded sheet can be deposited in the chamber.

This “gel extrusion” usually begins at the start of a run or even during the prerun. It is believed to be caused by a buildup of charge on the surface of the glass plate such that the gel is not bound to the plate after pouring. As the voltage is applied, the gel migrates toward the upper electrode.

The gel image can show a variety of anomalous effects, including catastrophic loss of resolution, lane splitting, extreme band tilt, and band distortion (Figure 7-56).

Almost all known cases of gel extrusion have been resolved by either acid washing or alcoholic KOH washing. Refer to the cleaning procedures on page 4-9.

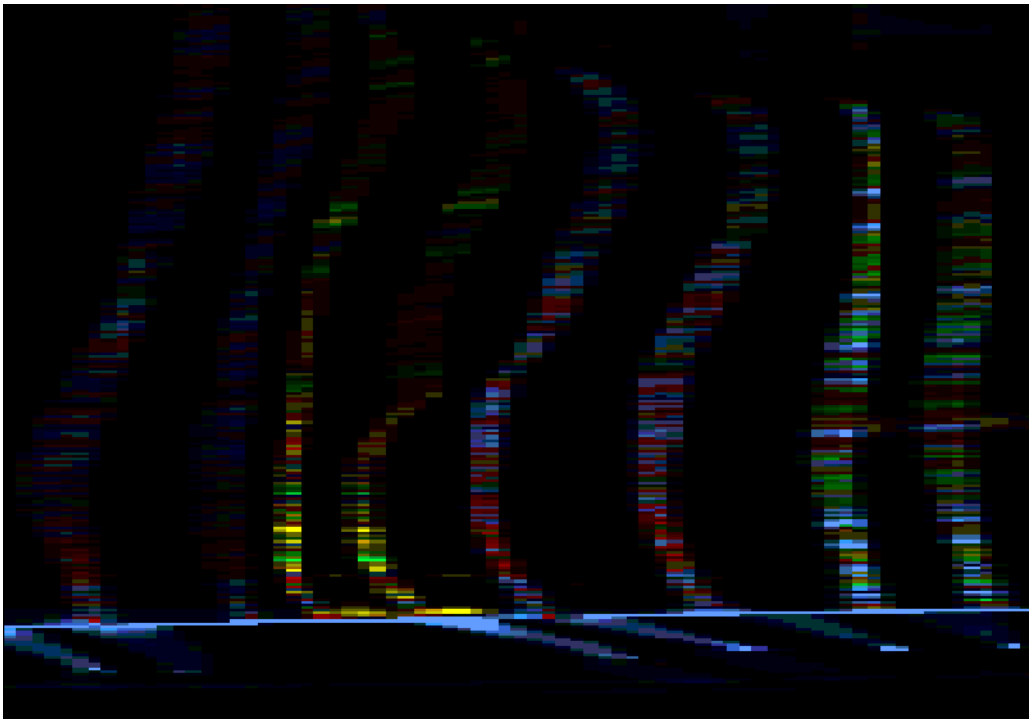


Figure 7-56 Effect of gel extrusion on sample migration

Temporary Loss of Signal

This problem usually occurs at the beginning of the gel (between 150 and 250 base pairs in the analyzed data). It manifests itself as a band of little or no signal across the entire width of the gel image (Figure 7-57). Temporary loss of signal has been traced to contaminants on the gel plates. These contaminants include surfactants, fatty acids, and long chain polymers that are not removed when the plates are washed.

Rinsing glass plates with hot deionized water (90 °C) has been found to remove the contaminants that cause temporary loss of signal. Refer to page 4-9 for more information.

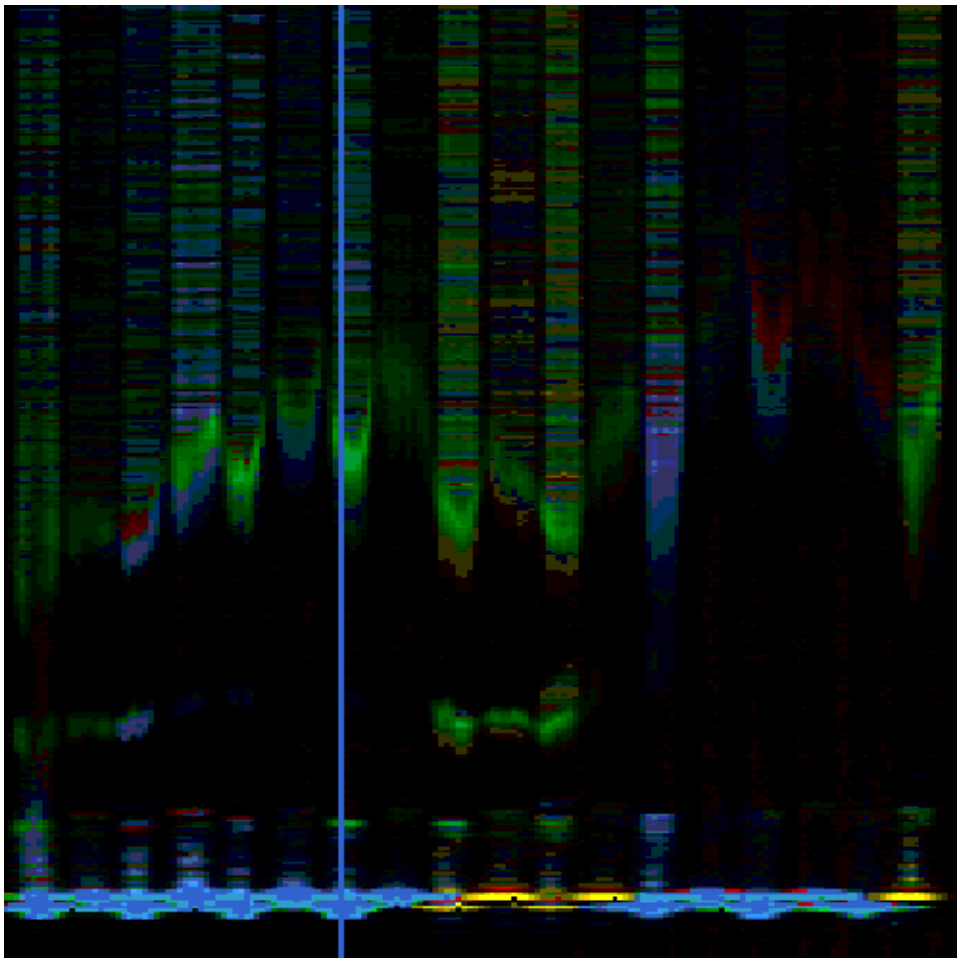


Figure 7-57 Portion of an ABI PRISM 377 gel image showing temporary loss of signal. The vertical blue line is from dust in the read region of the gel.

Poor-Quality Gel Plates

Plates from vendors other than Applied Biosystems may not have adequate quality control for use on our instruments. Several problems result from poor quality plates, including warping. Figure 7-58 shows data from plates that warped after 6 months of use.

When plates become warped, the laser is no longer focused correctly on the gel. When this occurs on the ABI 373 DNA Sequencer, laser light is scattered back to the detector, causing the gel image to appear blue and green and obscuring data. On the ABI PRISM 377 DNA Sequencer, there is a filter to keep out most of the scattered laser light. Data quality still suffers because the scattering results in less excitation of the dyes by the laser.

Always use high-quality gel plates.

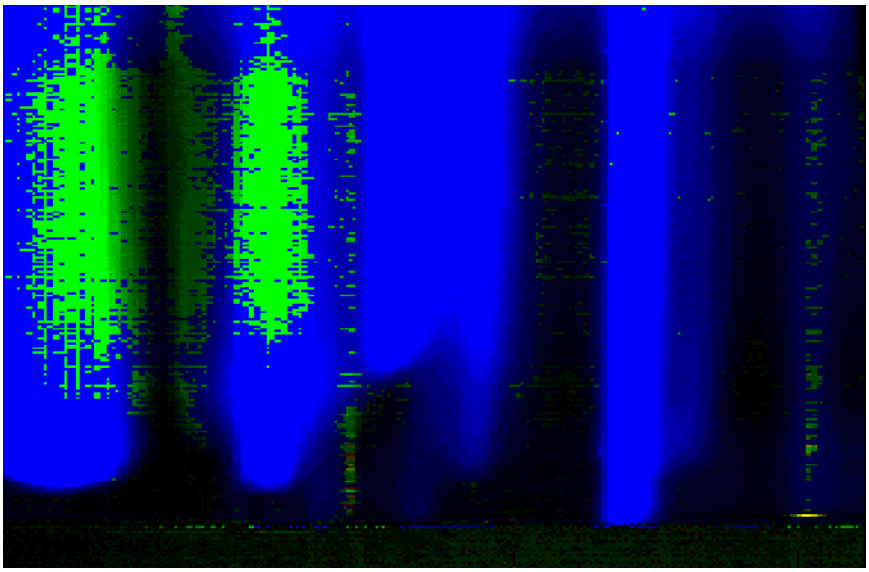


Figure 7-58 Effect of warped gel plates on ABI 373 sequencing data

Troubleshooting Gel Electrophoresis

Observation	Possible Causes	Recommended Actions
Gel runs too quickly	Total polymer concentration too low	Check reagents. Prepare new solutions using fresh reagents. **Note** Do not use TBE buffer if it has precipitate in it.
	Bisacrylamide concentration too low	
	Buffer concentration too high	
Gel runs too slowly	Total polymer concentration too high	Check reagents. Prepare new solutions using fresh reagents.
	Bisacrylamide concentration too high	
	Buffer concentration too low	
	Old gel	Use gels within 2–6 hours of casting for the ABI PRISM 377 DNA Sequencer. Use gels within 18–24 hours of casting for the ABI 373 DNA Sequencer. **IMPORTANT** Do not refrigerate.
Poor resolution caused by gel	Poor quality reagents, especially acrylamide (see Figure 7-50 on page 7-44), APS, and TEMED	Use fresh reagents from a reliable source.
	Small bubble between load and read region	Clean plates thoroughly. Cast gel carefully. Remove bubble by tapping plates while pouring.
	Well shape not flat	Assure that no air bubbles are trapped by casting comb at gel surface. Do not push the sharktooth comb too far into the gel.
	Old gel	Use gels within 2–6 hours of casting for the ABI PRISM 377 DNA Sequencer. Use gels within 18–24 hours of casting for the ABI 373 DNA Sequencer. **IMPORTANT** Do not refrigerate.
	Variation in spacers	Use spacers and comb sets that are equal thickness.
	Temperature of room, gel solution, or glass too warm or cool during polymerization	20–23 °C is optimal.
Visible non-homogeneity (Schlieren pattern or “swirl” in gel)	Excessive TEMED or APS	Check reagents. Prepare new solutions using fresh reagents.
	Temperature too high	Polymerize at 20–23 °C.
	Insufficient reagent mixing	Mix reagents gently, but thoroughly.

Troubleshooting Gel Electrophoresis *(continued)*

Observation	Possible Causes	Recommended Actions
Polymerization too slow (gels should polymerize within 15–20 minutes)	Excessive dissolved oxygen	Keep vacuum filter strength/time constant. Stir and pour gel gently. Filter and pour gel at 20–23 °C.
	Not enough TEMED or APS (or degraded)	Check reagents. Prepare new solutions using fresh reagents.
	Temperature too low during casting	Polymerize at 20–23 °C.
	Did not use deionized water	Use only deionized or distilled water for making all solutions.
Gel loses signal around 200 bp (see page 7-51)	Contaminant polymers on plate surface	Wash plates with mild detergent and hot deionized water rinses.
Lanes appear as smears	Impure or degraded TEMED or APS	Use fresh reagents.
	Samples are overloaded	Follow loading procedure.
	Electrophoresis failure due to buffer leak (see Figure 7-54 on page 7-48)	Make sure that the plates are clamped correctly, and that the upper buffer chamber gasket makes a proper seal. Do not spill buffer behind the upper buffer chamber, as wicking can occur.
Gel image contains vertical red streaks near end of run (top of gel image) ("red rain," see Figure 7-55 on page 7-49	Gel destruction in read region	Wrap the gel to prevent drying. Run at a lower temperature or voltage.
Gel image contains green/blue streaks throughout run (see Figure 7-52 on page 7-46)	Fluorescent contaminant in gel	Vacuum filter solution. Cast gel in dust-free environment.
	Urea crystals present in gel	Use room temperature reagents. Pour at 20–23 °C. **IMPORTANT** Do not refrigerate.
	Particles on outer surface of plates in read region	Wipe read region with damp lint-free KimWipe
Blue or green streaks ("curtain") at top of gel image (see Figure 7-53 on page 7-47)	Buffer leak	Make sure that the plates are clamped correctly, and that the upper buffer chamber gasket makes a proper seal. Do not spill buffer behind the upper buffer chamber, as wicking can occur.
Blue or green curtain obscuring entire gel image (see Figure 7-58 on page 7-52)	Warped gel plate	Use gel plates from Applied Biosystems.
Green streak through entire gel lane	Protein in template	Clean up the template before performing sequencing reactions.
Greenish-yellow haze	Poor gel plate alignment	Remove the gel plates and realign them correctly.
	Fluorescent contaminant in gel	Use fresh reagents. Do not write on the gel plates with marking pens.
	Residual detergent on plates	Rinse plates thoroughly with hot deionized water.

Troubleshooting Capillary Electrophoresis on the ABI PRISM 310

Overview

This section shows examples of problems that are specific to capillary electrophoresis. Refer to the table on page 7-57 for a more complete guide to troubleshooting capillary electrophoresis.

Capillary Failure

Figure 7-59 shows data from a capillary that had been used for more than 100 injections. Data is noisy and has trailing peaks.

We recommend that capillaries be replaced after 100 injections or when you start to see signs of capillary failure.

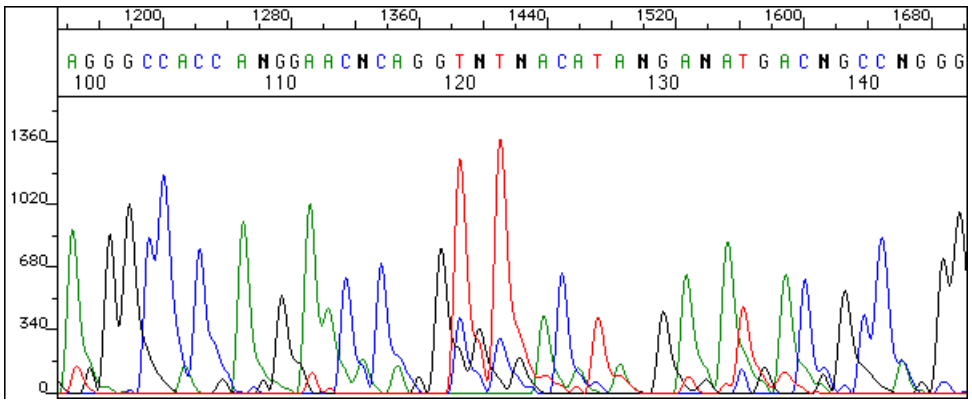


Figure 7-59 Trailing peaks caused by capillary failure

Spikes

Spikes are caused by particulate matter in the polymer passing through the detection window and scattering laser light.

Figure 7-60 on page 7-56 shows the effect of a spike on analyzed sequencing data from a BigDye primer reaction. The spike observed in the center of the panel is almost twofold higher than the sequencing peaks. The mobility shift algorithm makes this spike look as if the red portion precedes the blue portion, but in the raw data the spike is a single, four-color peak (Figure 7-61 on page 7-56).

If your data shows spikes:

- Clean the syringe and pump block with filtered, deionized water.
- Use a fresh capillary that has not been exposed to dust, *i.e.*, left on the benchtop.
- Filter the POP-6™ polymer with a 0.2-µm or 0.45-µm disk filter attached to a plastic syringe.

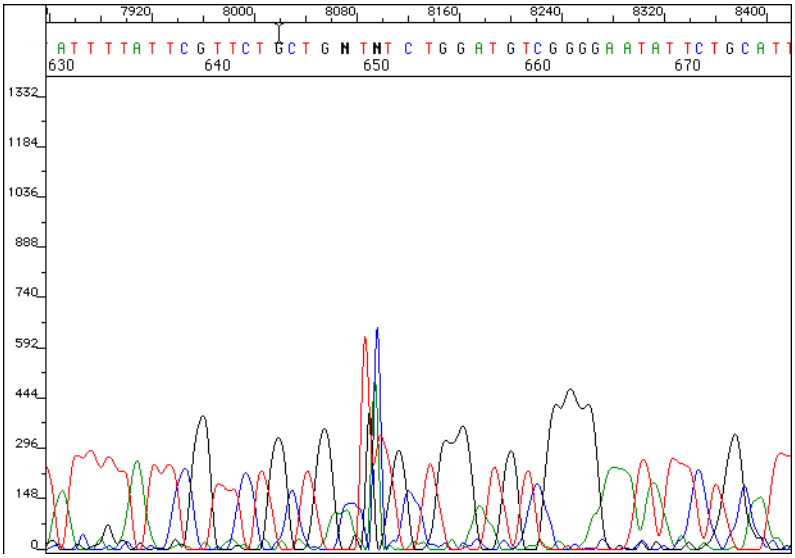


Figure 7-60 A spike in analyzed sequencing data

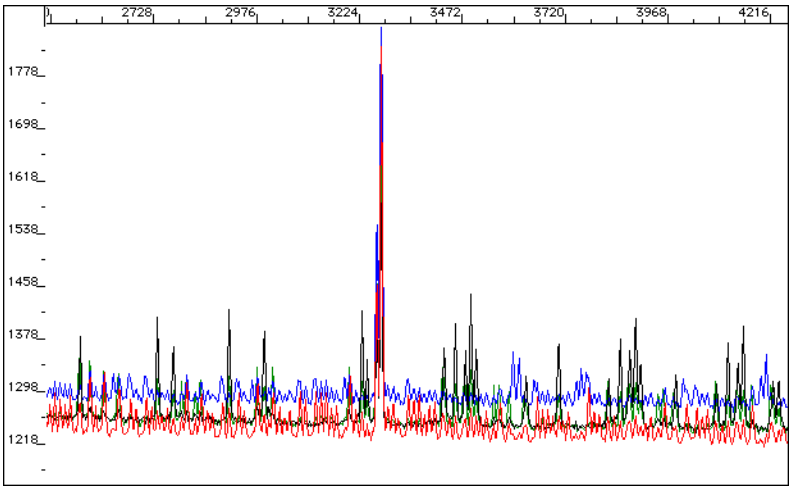


Figure 7-61 A spike in raw data

Troubleshooting Capillary Electrophoresis

Observation	Possible Causes	Recommended Actions
Data was not automatically analyzed	Sample Sheet not completed or completed incorrectly	Complete the Sample Sheet as described in your user's manual.
	Injection List not completed or completed incorrectly	Complete the Injection List as described in your user's manual.
	Analysis preferences set incorrectly in data collection program	Check the collection software preferences to make sure that Autoanalyze with Sequencing Analysis Software is selected under the Sequence Injection List Defaults.
	Insufficient free RAM	Restart the computer before collecting data. **Note** You should always restart the computer before collecting data.
	Conflicting extensions	Choose Extensions Manager from the Control Panels. Turn off any extensions that were not part of the original installation and restart computer.
No current	Too little or no buffer in anode buffer reservoir	Replenish buffer reservoir.
	Too little or no buffer in position 1 of autosampler	Replenish buffer in position 1 of autosampler.
	Electrode bent	Replace or straighten electrode and recalibrate autosampler.
	Capillary bent away from electrode	Tape capillary securely to heat plate to keep capillary from shifting position. Place the tape on the heat plate just above the electrode holder. Refer to the ABI PRISM 310 *Genetic Analyzer User's Manual*.
	Unfilled capillary or bubbles in capillary	Check system for leaks. Replace capillary if necessary and rerun module.
	Major leaks in system. Polymer does not enter capillary	Check system for leaks. **Note** Filling the capillary should cause the Gel Pump value in the Status Window to increase by only 1–2 steps. If the instrument detects a syringe leak, a warning message appears on the screen.
	Pump blockage (pump is plugged with urea or crystallized buffer)	Remove and clean pump block. Refer to the *ABI PRISM 310 Genetic Analyzer User's Manual.*
	Loose valve fittings or syringe	Tighten valve fittings and syringe.
	Anode buffer valve does not open	Open buffer valve. **Note** The valve should depress easily when you push the top with your finger tip. After you release the pressure the valve should spring to the "open" position. If the valve is stuck, it should be cleaned.
	Plugged, broken, or nonconducting capillary	Replace the capillary.
	Poor quality water in buffer solutions	Remake buffer with freshly autoclaved, distilled, deionized water
	Incorrect polymer solution formulation	Make or install new polymer solution

Troubleshooting Capillary Electrophoresis *(continued)*

Observation	Possible Causes	Recommended Actions
No current	Corrupted firmware	Resend firmware by performing a cold boot reset.
	Syringe Pump Force too low. Capillary is not being filled completely	Call DNA Technical Support.
Low current	Small bubble in capillary blocking current flow	Replenish gel in capillary.
	Small bubble in pump block	Remove bubble by repriming the pump block with polymer.
	Plugged, broken, or nonconducting capillary	Replace the capillary.
	Poor quality water in buffer solutions	Remake buffer with freshly autoclaved, distilled, deionized water.
	Old, defective, or incorrectly made buffer or polymer solution	Replace buffer or polymer solution.
Fluctuating current	Too little buffer in anodic jar	Replenish buffer jar.
	Small bubble in capillary blocking current flow	Replenish gel in capillary.
	Small bubble in pump block	Remove bubble by repriming the pump block with polymer.
	Broken or cracked capillary	Replace the capillary.
	Arcing to conductive surface on the instrument	Clean the hotplate and autosampler. Ensure that the ambient temperature is between 15 and 30 °C and the humidity is below 80%. Check for excessive condensation on the instrument.
	Position of electrode is not sufficiently below the buffer surface	Replenish buffer.
		Reposition electrode and recalibrate autosampler.
Current is normal at beginning of run and then decreases rapidly over the next several minutes	Loss of anodic buffer capacity	Replace the buffer.
Current too high	Decomposition of urea in polymer solution	Add fresh polymer solution to the syringe.
	Incorrect buffer formulation (most likely too concentrated)	Replace buffer with appropriate 1X running buffer.
	Arcing to conductive surface on the instrument	Clean the hotplate and autosampler. Ensure that the ambient temperature is between 15 and 30 °C and the humidity is below 80%. Check for excessive condensation on the instrument.
No signal	No sample added	Add sample.
	Sample not at bottom of tube	Spin sample tube in microcentrifuge.
	Air bubble at bottom of sample tube	Spin sample tube in microcentrifuge to remove air bubbles.
	Capillary misaligned with electrode	Align capillary and electrode. **Note** The capillary should be adjacent to, but not touching, the electrode. The capillary should protrude 0.5 mm past the electrode.

Troubleshooting Capillary Electrophoresis *(continued)*

Observation	Possible Causes	Recommended Actions
No signal	Capillary bent out of sample tube	Align capillary and electrode. Recalibrate autosampler. **Note** To verify whether a bent capillary is the problem, watch the movement of the autosampler tray during run operation.
	Autosampler not calibrated correctly	Calibrate autosampler in X, Y, and Z directions. **IMPORTANT** The capillary should almost touch the Z calibration point.
	Sealed sample tube septum (*i.e.*, septum will not open to allow electrode into sample tube)	Replace septum.
	Septum not placed in the sample tube properly	
Signal too low	Insufficient sample injected	Increase injection time.
	Old Template Suppression Reagent (TSR)	Use fresh TSR.
	Ions in sample (leading to insufficient sample injected)	Dialyze sample to remove ions.
	Sample not thoroughly mixed with TSR	Mix sample into TSR by pipetting up and down several times.
Signal too high	Too much sample injected into capillary.	Decrease injection time or injection voltage.
High baseline	Dirty capillary window	Clean capillary window with 95% ethanol.
	Capillary moved out of position in front of laser window	Position capillary in front of laser window.
	Precipitate in polymer	Allow polymer to equilibrate to room temperature before using.
		Use fresh polymer.
	Contaminant in polymer	Filter the polymer with a 0.2-µm or 0.45-µm disk filter attached to a plastic syringe.
	Incorrectly prepared and/or old buffer or polymer solutions	Replace buffer and polymer with fresh solutions.
	Improper filling of capillary	Run the Seq Fill Capillary run module to fill the capillary.
	Fluorescent contaminant in the capillary holder	Clean the capillary holder.
	Fluorescent contaminant in the sample	Purify the sample.
	Defective or old capillary	Replace the capillary.
	Matrix made incorrectly resulting in too much correction (also indicated by troughs under peaks)	Remake matrix. Be sure to: ♦ Remove the primer peak (or aberrant off-scale peaks) from the scan range. ♦ Pick the start and stop points on flat parts of the baseline when viewing raw data. ♦ Make the matrix using same polymer, buffer, and run conditions as sample injections.

Troubleshooting Capillary Electrophoresis *(continued)*

Observation	Possible Causes	Recommended Actions
Noisy baseline	Incorrectly prepared and/or old buffer or polymer solutions	Replace buffer and polymer with fresh solutions.
	Dirty capillary holder aperture	Clean the capillary holder.
	Defective capillary	Replace the capillary.
Spikes in baseline (see Figure 7-60 on page 7-56)	Precipitate in polymer	Allow polymer to equilibrate to room temperature before adding to capillary.
		Filter the polymer with a 0.2-µm or 0.45-µm disk filter attached to a plastic syringe.
		Use fresh polymer.
	Old polymer	Use fresh polymer.
Extra peaks in additional colors displayed underneath the position of one strong peak	Too much sample injected into capillary (indicated if any peak is greater than 4000 RFU)	Decrease injection time or injection voltage.
		Repeat using less DNA.
	Incorrect matrix chosen or poor matrix	Check matrix selection on Injection List. If correct, create a new matrix.
Extraneous peaks	Unincorporated dye primers or dye terminators (“dye blobs”)	Purify the extension products thoroughly before sequencing.
	Fluorescent contaminant in sample (often from marking pen ink)	Prepare new samples. Do not write on sample tubes or septa with marking pens.
	Renaturation of denatured samples	Load samples immediately following denaturation, or store on ice until you are ready to load. **IMPORTANT** Do not store samples on ice for more than 2 hours before loading.
	Dust or dirt in polymer (see Figure 7-60 on page 7-56)	Filter the polymer with a 0.2-µm or 0.45-µm disk filter attached to a plastic syringe.
Stop peak (strong peak in all four colors)	Secondary structure in sequence	Use dye terminator chemistries instead of dye primer chemistries.
	Primer-dimer (primer oligomerization) in PCR sequencing	Make sure that there is no sequence complementarity between the two PCR primers. Use a sequencing primer that is different from the two PCR primers. Ensure that the sequencing primer does not overlap the sequence of either PCR primer.
Poor base spacing	Incorrect dye set/primer file	Check dye set/primer file used.
	Incorrect polymer composition	Check urea concentration and polymer composition against protocol.
	Incorrect electrophoresis temperature	Check the Injection List for temperature setting. If correct on Injection List, check the Log for a recording of the actual electrophoresis temperature.
Inconsistent peak mobilities at beginning of run (*i.e.*, peaks come off at higher scan numbers in the first injection)	Capillary temperature not at equilibrium	Repeat the injection of the first sample. **Note** The run temperature can be set in the Manual Control window while the samples are being prepared, but we still recommend repeating the first sample.

Troubleshooting Capillary Electrophoresis *(continued)*

Observation	Possible Causes	Recommended Actions
Fragments migrate slower than normally	Capillary allowed to dry out	Leave capillary in buffer or water when not in use.
	Dirty sample	Purify extension reactions before sequencing.
	Air bubbles in pump block or capillary	Check for leaks and remove air bubbles.
	Incorrect buffer concentration	Remake running buffer.
	Clogged pump block	Remove pump block and clean it.
	Syringe pump failure	Call DNA Technical Support.
Runs get progressively slower (*i.e.*, primer peaks come off at higher and higher scan numbers)	Leaking syringe: polymer is not filling capillary before every injection	Clean syringe thoroughly.
		Replace syringe.
	Syringe out of polymer	Fill syringe with fresh polymer.
Runs get progressively faster (*i.e.*, primer peaks come off at lower and lower scan numbers)	Water in syringe	Prime syringe with small volume of polymer, invert syringe to coat capillary walls, and discard polymer. Then fill syringe with fresh running polymer.
Poor resolution (see Figure 7-59 on page 7-55)	Poor capillary performance	Replace capillary.
	Incorrectly prepared and/or old buffer or polymer solutions	Replace buffer and polymer with fresh solutions.
	Injection time too long (broad peaks)	Decrease injection time.
	Incorrectly prepared and/or degraded sample	Prepare new sample.
	Incorrect buffer formulation	Check if buffer formulation matches protocol requirements.
	Incorrect polymer composition	Check if polymer composition matches protocol requirements.
	Electrophoresis voltage too high	Decrease electrophoresis voltage by as much as 4 kV. **Note** Increase electrophoresis time accordingly.
	Sample concentrated by evaporation leaving excess salt behind	Do not concentrate sample by evaporation. Use an Amicon Centricon-100 column if necessary.
	Incomplete strand separation due to insufficient heat denaturation	Make sure the samples are heated at 95 °C for 2 minutes prior to loading onto autosampler.
	Wrong capillary	Verify that you are using the correct capillary (see Table 5-2 on page 5-8).
	Oil in sample (from DNA Thermal Cycler 480)	Carefully pipette sequencing reactions without oil carryover.
		Remove oil by organic extraction.
	Poor quality water	Use freshly autoclaved, distilled, deionized water.
	Syringe empty or incorrect Syringe Max Travel value	Fill syringe if necessary and recalibrate Syringe Max Travel value.

Troubleshooting Software Settings

Overview

This section shows examples of problems that can occur in sequencing data when software settings are incorrect.

Incorrect Run Module

Figure 7-62 shows data from a dRhodamine terminator sample, which should have been collected with a Filter Set E run module, but was collected using a Filter Set A run module.

Note One of the most common mistakes made with the new dRhodamine-based chemistries is to collect data using Filter Set A.

If you collect data using the wrong filter set, you should rerun the samples. If this is impossible, a matrix can be made from the data on the ABI PRISM 310 and ABI PRISM 377 instruments (see page 6-14). Data analyzed this way will not be free of multicomponenting noise.

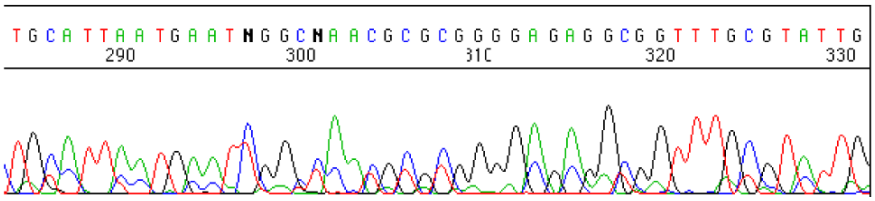


Figure 7-62 dRhodamine terminator sample run with a Filter Set A run module

Incorrect Dye Set/Primer (Mobility) File

If you analyze data with the wrong mobility file, the data can be reanalyzed with the correct mobility file as described in your user's manual.

Analyzing BigDye primer data with a mobility file for dRhodamine terminator or BigDye terminator chemistry (or vice versa) causes both shifted peaks and miscalled bases (Figure 7-63). These three chemistries use the same dyes for fluorescence emission, but on different bases. See Chapter 2, especially page 2-14, for more information.

Figure 7-63 shows BigDye terminator data analyzed with a BigDye primer mobility file. The C, G, and T bases are called incorrectly, and the As overlap with the following peaks. Figure 7-64 on page 7-63 shows the same sample file reanalyzed with the correct mobility file.

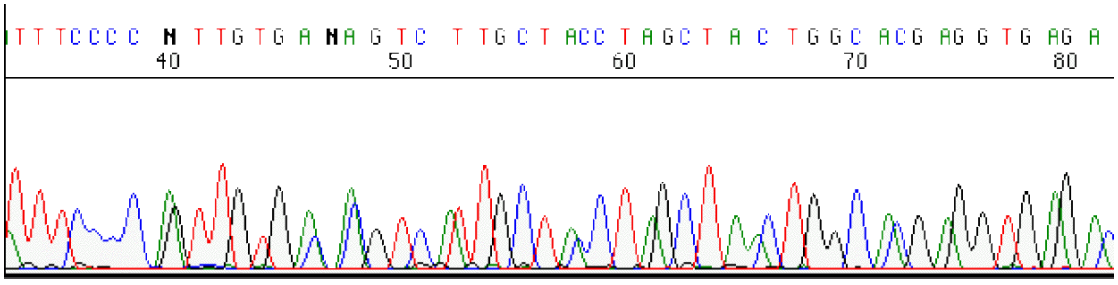


Figure 7-63 BigDye terminator data analyzed with a BigDye primer dye set/primer (mobility) file

Note Mobility shifts and dye set/primer file names for the dRhodamine Terminators are similar to those for the BigDye Terminators. If a mobility file for the wrong sequencing chemistry is used, C and T bases will be miscalled because of differences in which terminators are labeled with which dyes (see page 2-14).

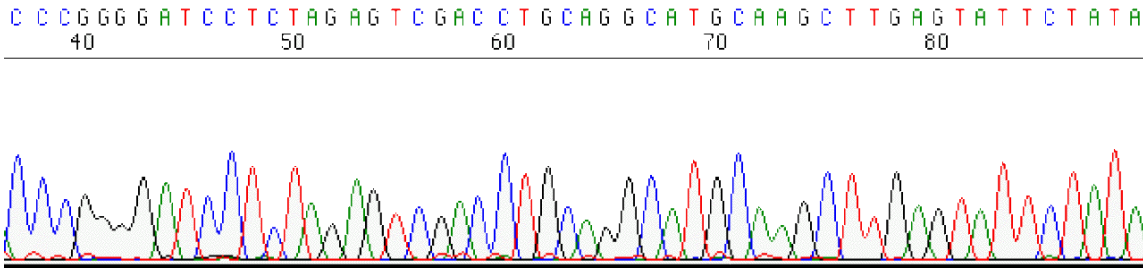


Figure 7-64 BigDye terminator data analyzed with the correct dye set/primer file

Incorrect or Poor-Quality Instrument (Matrix) File

If you use the wrong instrument file (but the correct run module), the data can be reanalyzed with the correct instrument file. Figure 7-65 shows BigDye terminator data, collected on an ABI PRISM 377 DNA Sequencer, with specific peaks under peaks throughout the run. For example, every black peak has a smaller red peak underneath it.

An incorrect instrument file, one for Filter Set A instead of Filter Set E, was used to analyze the data. If your data looks like this, you should check that the correct run module was used to collect the data. If the correct run module and instrument file were used, you may have a poor quality matrix. In this case, the instrument file should be remade (see page 6-7). Figure 7-66 on page 7-64 shows the same data analyzed with the correct instrument file.

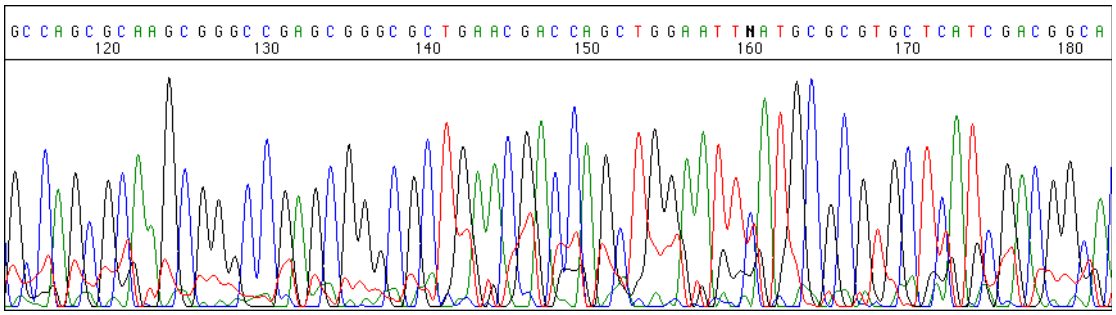


Figure 7-65 BigDye terminator data collected with a Filter Set E run module and analyzed with an incorrect (Filter Set A) instrument file

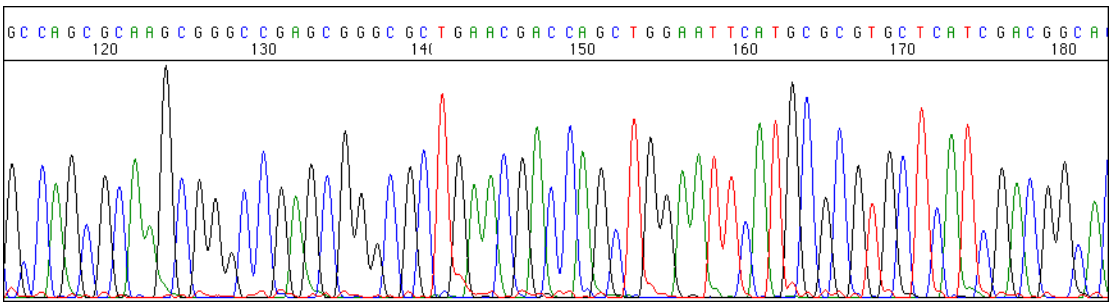


Figure 7-66 BigDye terminator data collected with a Filter Set E run module and analyzed with the correct (Filter Set E) instrument file

Instrument files do not change over time, but instruments do change. You may need to remake the instrument file as the filter wheel (on an ABI 373 DNA Sequencer) ages or if any changes to the optics occur, *e.g.*, the CCD camera (on an ABI PRISM 310 or ABI PRISM 377 instrument) is replaced.

Figure 7-67 shows BigDye terminator data analyzed with a poor matrix file. Bad multicomponenting is characterized by specific peaks under peaks throughout the run, in this case, smaller red peaks under blue peaks.

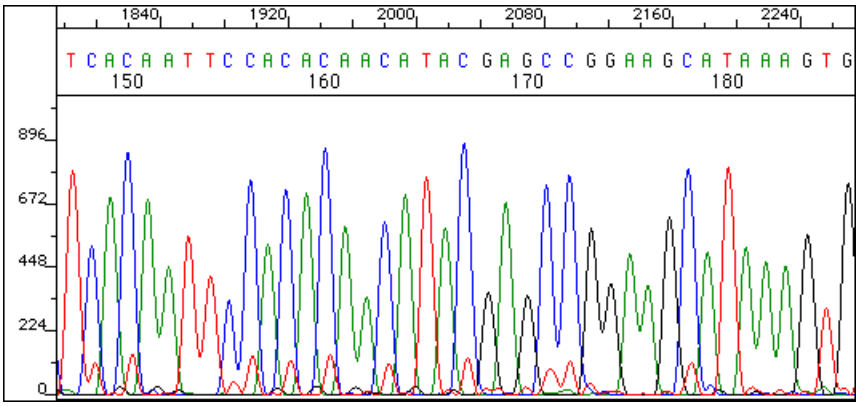


Figure 7-67 BigDye terminator data analyzed with a poor Filter Set E matrix file

See your instrument user's manual for instructions on creating a new instrument file or follow the instructions on page 6-8 for creating an instrument file for Filter Set E (dRhodamine-based chemistries).

Incorrect Peak 1 Location

During analysis, the software assigns the Peak 1 Location and the Start Point to the same scan number. Occasionally the value assigned is incorrect. This happens more commonly with dye terminator chemistries than with dye primer chemistries. Correct assignment of the Peak 1 Location value is important for mobility corrections to be applied properly.

With dye primer chemistries the large primer peak helps the software to assign the Peak 1 Location and Start Point values. With dye terminator chemistries, there is no primer peak, so the software must detect the small sequence data peaks.

If these sequence peaks are obscured by peaks from unincorporated dye terminators and/or other fluorescent peaks are present before the start of the real sequence, the software can assign the Peak 1 location and Start Point too early (Figure 7-68 on

page 7-65). If the sequence data is weak, the Peak 1 location and Start Point can be assigned too late.

See “Setting the Data Analysis Range” on page 6-15 for the procedures to change the Peak 1 Location and Start Point.

Large peaks can also cause problems in scaling the analyzed data. For example, in Figure 7-68 a very large red (T) peak at base 130 resulted in all other red peaks appearing very small.

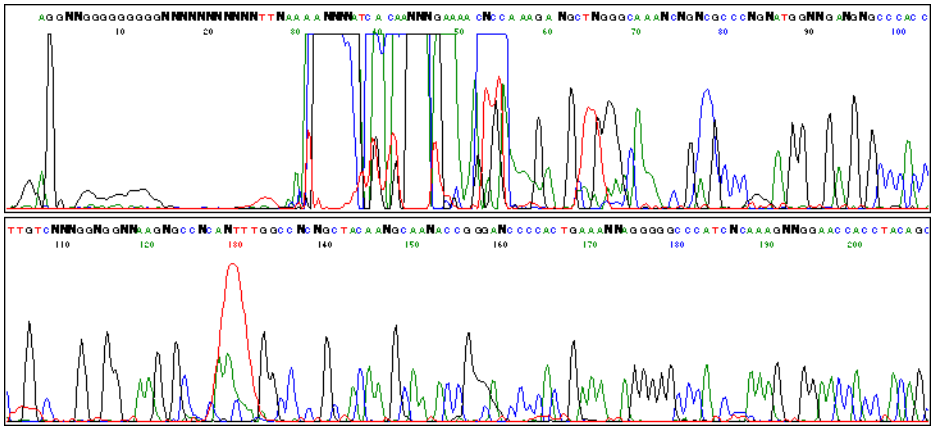


Figure 7-68 Incorrect Peak 1 Location and Start Point (both set to 963 scans)

In this case the Start Point can be set after the terminator peaks and the data reanalyzed. When this was done, the T peaks were scaled normally (Figure 7-69).

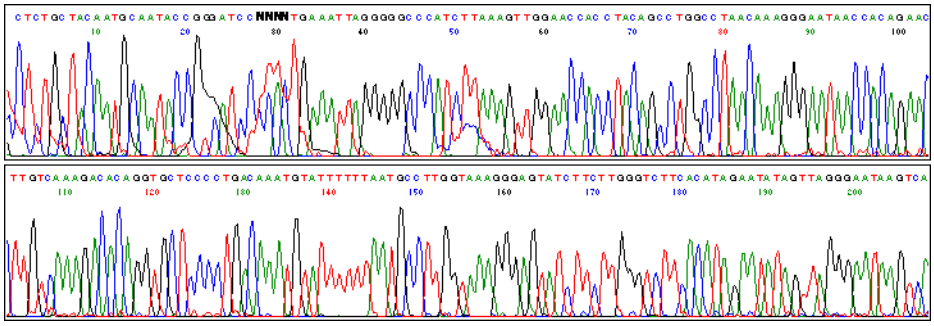


Figure 7-69 Better Peak 1 Location (1260 scans) and Start Point (2020 scans)

In this example, the Start Point value is greater than the Peak 1 Location value. The Peak 1 Location value should still be set to the beginning of the sequence. This ensures that the mobility corrections and spacing are applied properly.

Gel Preparation

A

Introduction

Gel Formulations

There are several choices of sequencing gel formulations for 34- and 36-cm well-to-read (wtr) lengths. The 29:1 polyacrylamide, Long Ranger™, and PAGE-PLUS gels perform similarly on both the ABI™ 373 and ABI PRISM® 377 instruments. These gels generally perform better than 19:1 polyacrylamide gels.

For 48-cm gels, the 5.25% PAGE-PLUS gels generally provide the longest read lengths.

For 2400-scan/hr runs on the ABI PRISM 377 DNA Sequencer, the best gel to use is the 4.5% 29:1 polyacrylamide. This type of gel performs the best under these extreme run conditions.

Different laboratories may obtain different results. We recommend testing several gel formulations to see which works best under your particular run conditions.

Table A-1 lists recommended gel formulations for the ABI 373 and ABI PRISM 377 DNA Sequencers.

Table A-1 Gel Formulations

	34-cm or 36-cm well-to-read (wtr)		48-cm well-to-read (wtr)	
Instrument	**Gel Type**	**Volume (mL)**	**Gel Type**	**Volume (mL)**
ABI 373	6% 19:1 polyacrylamide[a]	80		
	4.75% 19:1 polyacrylamide	80	4% 19:1 polyacrylamide	100
	5% 29:1 polyacrylamide	80	4.25% 29:1 polyacrylamide	100
	5.75% Long Ranger	80	5% Long Ranger	100
	5.75% PAGE-PLUS	80	5.25% PAGE-PLUS	100
ABI PRISM 377	4% 19:1 polyacrylamide	50	4% 19:1 polyacrylamide	50
	4.5% 29:1 polyacrylamide	50	4.25% 29:1 polyacrylamide	50
	5% Long Ranger	50	4.75% Long Ranger	50
	4.8% PAGE-PLUS	50	5.25% PAGE-PLUS	50

a. Used for 24-cm well-to-read gels.

Protocol and Run Conditions for 19:1 Polyacrylamide Gels

Preparing 40% Acrylamide Stock (19:1)

Step	Action
1	Working in a fume hood, combine the following acrylamide and bisacrylamide in a glass beaker: ♦ Acrylamide, 57 g ♦ Bisacrylamide, 3 g **! WARNING ! CHEMICAL HAZARD. Acrylamide and bisacrylamide are poisons, neurotoxins, irritants, carcinogens, and possible teratogens. Acrylamide and bisacrylamide sublime (the solids release toxic vapor) and are harmful if swallowed, inhaled, or absorbed through the skin. Effects are cumulative. When handling, always wear protective equipment (lab coat, safety glasses, and chemical-resistant gloves) and use in a well-ventilated area. On a routine basis, thoroughly clean surfaces subject to contamination.**
2	Dissolve the crystalline acrylamide and bisacrylamide in sufficient distilled, deionized water to bring the total volume to 135 mL.
3	Add 15 g of mixed-bed, ion-exchange resin.
4	Stir at room temperature until all crystals dissolve. Continue stirring for 5–10 minutes.
5	Filter the mixture through a 0.2-µm cellulose nitrate filter.
6	Transfer the filtrate to a graduated cylinder and bring the total volume to 150 mL with distilled, deionized water.
7	Store at 2–6 °C.

Note 40% acrylamide stock lasts for 1 month at 2–6 °C.

Ingredients and Run Conditions for the ABI PRISM 377

For 36-cm and 48-cm WTR Runs—4% 19:1 Polyacrylamide Gel, 6 M Urea

Ingredient	For 50 mL	Run Conditions
urea	18.0 g	♦ For 1200 scans/hr runs, use standard 36-cm 1200 scans/hr run modules. ♦ For 2400 scans/hr runs, use standard 36-cm 2400 scans/hr run modules. ♦ For 48-cm gels, use standard 48-cm, 1200 scans/hr run modules.
40% acrylamide stock	5.0 mL	
deionized water	25 mL	
Mixed-bed ion exchange resin	0.5 g	
Filter and degas the above ingredients before adding TBE.		
10X TBE	5.0 mL	
10% APS	250 µL	
TEMED	35 µL	
Bring to final volume (50 mL) with deionized water.		

Ingredients and Run Conditions for the ABI 373

For 24-cm WTR Runs—6% 19:1 Polyacrylamide Gel, 8.3 M Urea

Ingredient	For 80 mL	Run Conditions
urea	40.0 g	Use the standard run time of 14 hours.
40% acrylamide stock	12 mL	
deionized water	27 mL	
Mixed-bed ion exchange resin	0.5 g	
Filter and degas the above ingredients before adding TBE.		
10X TBE	8.0 mL	
10% APS	400 µL	
TEMED	45 µL	
Bring to final volume (80 mL) with deionized water.		

For 34-cm WTR Runs—4.75% 19:1 Polyacrylamide Gel, 8.3 M Urea

Ingredient	For 80 mL	Run Conditions
urea	40.0 g	Use the standard run time of 14 hours.
40% acrylamide stock	9.5 mL	
deionized water	27 mL	
Mixed-bed ion exchange resin	0.5 g	
Filter and degas the above ingredients before adding TBE.		
10X TBE	8.0 mL	
10% APS	400 µL	
TEMED	45 µL	
Bring to final volume (80 mL) with deionized water.		

For 48-cm WTR Runs—4% 19:1 Polyacrylamide Gel, 8.3 M Urea

Ingredient	For 100 mL	Run Conditions
urea	50.0 g	Use the standard run time of 18 hours.
40% acrylamide stock	10 mL	
deionized water	37 mL	
Mixed-bed ion exchange resin	0.5 g	
Filter and degas the above ingredients before adding TBE.		
10X TBE	10 mL	
10% APS	500 µL	
TEMED	50 µL	
Bring to final volume (100 mL) with deionized water.		

Preparing 19:1 Polyacrylamide Gels

Preliminary gel preparation steps:

Step	Action
1	Referring to the appropriate list of ingredients above and the *373 DNA Sequencing System User's Manual* or ABI PRISM 377 *DNA Sequencer User's Manual,* gather all the necessary lab equipment and ingredients.
2	Prepare all stock solutions per the list of ingredients.
3	For the ABI PRISM 377 DNA Sequencer: clean the gel plates thoroughly and mount them in the gel pouring cassette (or alternative device). For the ABI 373 DNA Sequencer: clean the gel plates thoroughly and prepare them for gel pouring.

Preparing the acrylamide–urea solution:

Step	Action
1	Combine urea, 40% acrylamide stock, deionized water, and mixed-bed ion exchange resin in a 150-mL beaker. **! WARNING ! CHEMICAL HAZARD. Urea causes eye, skin, and respiratory irritation. Lab experiments have shown mutagenic effects. Avoid contact. Wear chemical resistant gloves, safety goggles, and other protective clothing.** **! WARNING ! CHEMICAL HAZARD. Acrylamide and bisacrylamide are neurotoxins. Avoid inhalation and skin contact. Wear gloves at all times, and work in a fume hood when handling acrylamide solutions. Use appropriate precautions to avoid inhalation of crystalline acrylamide. Read the manufacturer's MSDS before handling.**
2	Stir the solution until all the urea crystals have dissolved.
3	Filter the solution through a 0.2-µm cellulose nitrate filter.
4	Degas for 2–5 minutes. **Note** Degas time for all gels should be constant to ensure a reproducible polymerization rate for all gels.
5	Transfer the solution to a 100-mL graduated cylinder.
6	Add filtered 10X TBE buffer. **IMPORTANT** Always remove the mixed-bed ion exchange resin by filtration (step 3 above) before adding the TBE buffer. Resin will destroy the effectiveness of the buffer.
7	Add deionized water to make the final volume: ♦ 50 mL for a 36-cm or 48-cm gel for the ABI PRISM 377 DNA Sequencer ♦ 80 mL for a 24-cm or 34-cm gel for the ABI 373 DNA Sequencer ♦ 100 mL for a 48-cm gel for the ABI 373 DNA Sequencer **IMPORTANT** If the plates are not clean and ready for gel pouring, prepare them before adding the polymerizing agents to your solution.

Adding the polymerizing reagents:

Step	Action
1	Add freshly made 10% APS, and swirl carefully to mix without introducing air bubbles. **Note** Be as accurate and reproducible as possible when making the 10% APS solution. Significant variation in this reagent can produce changes in data quality.
2	Add TEMED, and swirl carefully to mix without introducing air bubbles. **! WARNING ! CHEMICAL AND FIRE HAZARD. TEMED is extremely flammable and can be very destructive to the skin, eyes, nose, and respiratory system. Keep it in a tightly closed container. Avoid inhalation and contact with skin, eyes and clothing. Always work under a hood and wear chemical resistant gloves when handling TEMED solutions. Read the MSDS in the Safety Summary included with your instrument user's manual.**
3	Cast the gel using one of the methods described in your instrument user's manual. **Note** Allow the gel to polymerize for a minimum of 2 hours.

Protocol and Run Conditions for 29:1 Polyacrylamide Gels

Preparing 40% Acrylamide Stock (29:1)

Step	Action
1	Working in a fume hood, combine the following amounts of acrylamide and bisacrylamide in a glass beaker: ♦ Acrylamide, 58 g ♦ Bisacrylamide, 2 g **! WARNING ! CHEMICAL HAZARD. Acrylamide and bisacrylamide are poisons, neurotoxins, irritants, carcinogens, and possible teratogens. Acrylamide and bisacrylamide sublime (the solids release toxic vapor) and are harmful if swallowed, inhaled, or absorbed through the skin. Effects are cumulative. When handling, always wear protective equipment (lab coat, safety glasses, and chemical-resistant gloves) and use in a well-ventilated area. On a routine basis, thoroughly clean surfaces subject to contamination.**
2	Dissolve the crystalline acrylamide and bisacrylamide in sufficient distilled, deionized water to bring the total volume to 135 mL.
3	Add 15 g of mixed-bed, ion-exchange resin.
4	Stir at room temperature until all crystals dissolve. Continue stirring for 5–10 minutes.
5	Filter the mixture through a 0.2-µm cellulose nitrate filter.
6	Transfer the filtrate to a graduated cylinder and bring the total volume to 150 mL with distilled, deionized water.
7	Store at 2–6 °C.

Note 40% acrylamide stock lasts for 1 month at 2–6 °C.

Ingredients and Run Conditions for the ABI PRISM 377

For 36-cm WTR Runs—4.5% 29:1 Polyacrylamide Gel, 6 M Urea

This is the best formulation to use for 2400 scans/hr runs.

Ingredient	For 50 mL	Run Conditions
urea	18.0 g	For 1200 scans/hr runs: ♦ Use standard 36-cm 1200 scans/hr run modules. ♦ Increase run time to 9 hours. For 2400 scans/hr runs: ♦ Use standard 36-cm 2400 scans/hr run modules. ♦ Increase run time to 4 hours.
40% acrylamide stock	5.63 mL	
deionized water	25 mL	
Mixed-bed ion exchange resin	0.5 g	
Filter and degas the above ingredients before adding TBE.		
10X TBE	5.0 mL	
10% APS	250 µL	
TEMED	30 µL	
Bring to final volume (50 mL) with deionized water.		

For 48-cm WTR Runs—4.25% 29:1 Polyacrylamide Gel, 6 M Urea

Ingredient	For 50 mL	Run Conditions
urea	18.0 g	♦ Use standard 48-cm run modules. ♦ Increase run time to 11 hours.
40% acrylamide stock	5.31 mL	
deionized water	25 mL	
Mixed-bed ion exchange resin	0.5 g	
Filter and degas the above ingredients before adding TBE.		
10X TBE	5.0 mL	
10% APS	250 µL	
TEMED	30 µL	
Bring to final volume (50 mL) with deionized water.		

Ingredients and Run Conditions for the ABI 373

For 34-cm WTR Runs—5% 29:1 Polyacrylamide Gel, 8.3 M Urea

Ingredient	For 80 mL	Run Conditions
urea	40.0 g	Increase run time to 18 hours.
40% acrylamide stock	10 mL	
deionized water	37 mL	
Mixed-bed ion exchange resin	0.5 g	
Filter and degas the above ingredients before adding TBE.		
10X TBE	8.0 mL	
10% APS	400 µL	
TEMED	48 µL	
Bring to final volume (80 mL) with deionized water.		

For 48-cm WTR Runs—4.25% 29:1 Polyacrylamide Gel, 7 M Urea

This formulation has not been tested in Applied Biosystems laboratories, but has been used successfully in several customer laboratories.

Ingredient	For 100 mL	Run Conditions
urea	42 g	Run at 40 W for 17 hours.
40% acrylamide stock	10.65 mL	
deionized water	44 mL	
Mixed-bed ion exchange resin	0.5 g	
Filter and degas the above ingredients before adding TBE.		
10X TBE	10 mL	
10% APS	500 µL	
TEMED	70 µL	
Bring to final volume (100 mL) with deionized water.		

Preparing 29:1 Polyacrylamide Gels

Preliminary gel preparation steps:

Step	Action
1	Referring to the appropriate list of ingredients above and the *373 DNA Sequencing System User's Manual* or ABI PRISM 377 *DNA Sequencer User's Manual,* gather all the necessary lab equipment and ingredients.
2	Prepare all stock solutions per the list of ingredients.
3	For the ABI PRISM 377 DNA Sequencer: clean the gel plates thoroughly and mount them in the gel pouring cassette (or alternative device). For the ABI 373 DNA Sequencer: clean the gel plates thoroughly and prepare them for gel pouring.

Preparing the acrylamide–urea solution:

Step	Action
1	Combine urea, 40% acrylamide stock, deionized water, and mixed-bed ion exchange resin in a 150-mL beaker. **! WARNING ! CHEMICAL HAZARD. Urea causes eye, skin, and respiratory irritation. Lab experiments have shown mutagenic effects. Avoid contact. Wear chemical resistant gloves, safety goggles, and other protective clothing.** **! WARNING ! CHEMICAL HAZARD. Acrylamide and bisacrylamide are neurotoxins. Avoid inhalation and skin contact. Wear gloves at all times, and work in a fume hood when handling acrylamide solutions. Use appropriate precautions to avoid inhalation of crystalline acrylamide. Read the manufacturer's MSDS before handling.**
2	Stir the solution until all the urea crystals have dissolved.
3	Filter the solution through a 0.2-µm cellulose nitrate filter.

Preparing the acrylamide–urea solution: *(continued)*

Step	Action
4	Degas for 2–5 minutes. **Note** Degas time for all gels should be constant to ensure a reproducible polymerization rate for all gels.
5	Transfer the solution to a 100-mL graduated cylinder.
6	Add filtered 10X TBE buffer. **IMPORTANT** Always remove the mixed-bed ion exchange resin by filtration (step 3 above) before adding the TBE buffer. Resin will destroy the effectiveness of the buffer.
7	Add deionized water to make the final volume: ♦ 50 mL for a 36-cm or 48-cm gel for the ABI PRISM 377 DNA Sequencer ♦ 80 mL for a 34-cm gel for the ABI 373 DNA Sequencer ♦ 100 mL for a 48-cm gel for the ABI 373 DNA Sequencer **IMPORTANT** If the plates are not clean and ready for gel pouring, prepare them before adding the polymerizing agents to your solution.

Adding the polymerizing reagents:

Step	Action
1	Add freshly made 10% APS, and swirl carefully to mix without introducing air bubbles. **Note** Be as accurate and reproducible as possible when making the 10% APS solution. Significant variation in this reagent can produce changes in data quality.
2	Add TEMED, and swirl carefully to mix without introducing air bubbles. **! WARNING ! CHEMICAL AND FIRE HAZARD. TEMED is extremely flammable and can be very destructive to the skin, eyes, nose, and respiratory system. Keep it in a tightly closed container. Avoid inhalation and contact with skin, eyes and clothing. Always work under a hood and wear chemical resistant gloves when handling TEMED solutions. Read the MSDS in the Safety Summary included with your instrument user's manual.**
3	Cast the gel using one of the methods described in your instrument user's manual. **Note** 29:1 polyacrylamide gels take a minimum of 2 hours to polymerize, sometimes longer.

Protocol and Run Conditions for Long Ranger and PAGE-PLUS Gels

Ingredients and Run Conditions for the ABI PRISM 377

For 36-cm WTR Runs

5.0% Long Ranger Gel, 6 M Urea

Ingredient	For 50 mL	Run Conditions
urea	18.0 g	For 1200 scans/hr runs: ♦ Use standard 36-cm 1200 scans/hr run modules. ♦ Increase run time to 9 hours. For 2400 scans/hr runs, use standard 36-cm 2400 scans/hr run modules.
50% gel stock solution	5.0 mL	
10X TBE	5.0 mL	
deionized water	to 50 mL	
10% APS	250 µL	
TEMED	25 µL	

4.8% PAGE-PLUS Gel, 6 M Urea

Ingredient	For 50 mL	Run Conditions
urea	18.0 g	For 1200 scans/hr runs: ♦ Use standard 36-cm 1200 scans/hr run modules. ♦ Increase run time to 9 hours. For 2400 scans/hr runs, use standard 36-cm 2400 scans/hr run modules.
40% gel stock solution	6.0 mL	
10X TBE	5.0 mL	
deionized water	to 50 mL	
10% APS	300 µL	
TEMED	30 µL	

For 48-cm WTR Runs

This is the best formulation to use for 48-cm gels.

5.25% PAGE-PLUS Gel, 6 M Urea

Ingredient	For 50 mL	Run Conditions
urea	18.0 g	♦ Use standard 48-cm run modules. ♦ Increase run time to 12 hours.
40% gel stock solution	6.6 mL	
10X TBE	5.0 mL	
deionized water	to 50 mL	
10% APS	250 µL	
TEMED	25 µL	

4.75% Long Ranger Gel, 6 M Urea

Ingredient	For 50 mL	Run Conditions
urea	18.0 g	♦ Use standard 48-cm run modules. ♦ Increase run time to 11 hours.
50% gel stock solution	4.75 mL	
10X TBE	5.0 mL	
deionized water	to 50 mL	
10% APS	250 µL	
TEMED	25 µL	

Preparing PAGE-PLUS and Long Ranger Gels for the ABI PRISM 377

Preliminary gel preparation steps:

Step	Action
1	Referring to the appropriate list of ingredients above, and your user's manual, gather all the necessary equipment and ingredients.
2	Prepare all stock solutions per the appropriate list of ingredients above.
3	Clean the gel plates thoroughly and mount them in the gel pouring cassette (or alternative device).

To prepare 5.0% Long Ranger, and 4.8 and 5.25% PAGE-PLUS gels:

Step	Action
1	Weigh out the urea and carefully transfer it to a stoppered, graduated cylinder.
2	Using a pipette, add the appropriate amount of gel stock solution and 10X TBE buffer to the cylinder.
3	Adjust the volume to 45 mL by slowly adding deionized water, and tapping the cylinder to release air bubbles trapped by the urea.
4	Stopper the cylinder and invert to dissolve the urea.
5	Allow the solution to warm to room temperature.
6	Add deionized water to make the final volume (50 mL).
7	Stopper the cylinder and mix the contents thoroughly.
8	Filter the solution through a 0.2-µm cellulose nitrate filter.
9	Degas for 2–5 minutes, and transfer the solution to a wide-mouthed container. **Note** Degas time for all gels should be constant to ensure a reproducible polymerization rate for all gels.

Adding the polymerizing reagents:

Step	Action
1	**IMPORTANT** If the plates are not clean and mounted in the gel cassette or other device, clean and mount them now before adding the polymerizing agents to your solution.
2	Add freshly made 10% APS, and swirl carefully to mix without introducing air bubbles. **Note** Be as accurate and reproducible as possible when making the 10% APS solution. Significant variation in this reagent can produce changes in data quality.
3	Add TEMED, and swirl carefully to mix without introducing air bubbles.
4	Cast the gel using one of the methods described in the ABI PRISM 377 *DNA Sequencer User's Manual*.
5	Allow the gel to polymerize for 2 hours before using.

Ingredients and Run Conditions for the ABI 373

For 34-cm WTR Runs

5.75% Long Ranger Gel, 8.3 M Urea

Ingredient	For 80 mL	Run Conditions
urea	40.0 g	Use the standard run time of 14 hours.
50% gel stock solution	9.2 mL	
10X TBE	8.0 mL	
deionized water	to 80 mL	
10% APS	400 µL	
TEMED	40 µL	

5.75% PAGE-PLUS Gel, 8.3 M Urea

Ingredient	For 80 mL	Run Conditions
urea	40.0 g	Use the standard run time of 14 hours.
40% gel stock solution	11.5 mL	
10X TBE	8.0 mL	
deionized water	to 80 mL	
10% APS	480 µL	
TEMED	48 µL	

For 48-cm WTR Runs

5.0% Long Ranger Gel, 8.3 M Urea

Ingredient	For 100 mL	Run Conditions
urea	50 g	Use the standard run time of 18 hours.
50% gel stock solution	10 mL	
10X TBE	10 mL	
deionized water	to 100 mL	
10% APS	500 µL	
TEMED	50 µL	

5.25% PAGE-PLUS Gel, 8.3 M Urea

Ingredient	For 100 mL	Run Conditions
urea	50 g	Use the standard run time of 18 hours.
40% gel stock solution	13.2 mL	
10X TBE	10.0 mL	
deionized water	to 100 mL	
10% APS	500 µL	
TEMED	50 µL	

Preparing PAGE-PLUS and Long Ranger Gels for the ABI 373

Preliminary gel preparation steps:

Step	Action
1	Referring to the appropriate list of ingredients above, and your user's manual, gather all the necessary equipment and ingredients.
2	Prepare all stock solutions per the appropriate list of ingredients above.
3	Clean the gel plates thoroughly and prepare them for gel pouring.

To prepare 5.0% Long Ranger, and 4.8 and 5.25% PAGE-PLUS gels:

Step	Action
1	Weigh out the urea and carefully transfer it to a 250-mL, stoppered, graduated cylinder.
2	Using a pipette, add the appropriate amount of gel stock solution and 10X TBE buffer to the cylinder.
3	Adjust the volume to 75 mL by slowly adding deionized water. Tap the cylinder to release air bubbles trapped by the urea.
4	Stopper the cylinder and invert to dissolve the urea.
5	Allow the solution to warm to room temperature.
6	Add deionized water to make the final volume: ♦ 80 mL for 34-cm gels ♦ 100 mL for 48-cm gels
7	Stopper the cylinder and mix the contents thoroughly.
8	Filter the solution through a 0.2-µm cellulose nitrate filter.
9	Degas for 2–5 minutes, and transfer the solution to a wide-mouthed container. **Note** Degas time for all gels should be constant to ensure a reproducible polymerization rate for all gels.

Adding the polymerizing reagents:

Step	Action
1	**IMPORTANT** If the plates are not clean and ready for gel pouring, prepare them before adding the polymerizing agents to your solution.
2	Add freshly made 10% APS, and swirl carefully to mix without introducing air bubbles. **Note** Be as accurate and reproducible as possible when making the 10% APS solution. Significant variation in this reagent can produce changes in data quality.
3	Add TEMED, and swirl carefully to mix without introducing air bubbles.
4	Cast the gel using one of the methods described in the *373 DNA Sequencing System User's Manual*.
5	Allow the gel to polymerize for 2 hours before using.

Preparing APS, TBE Buffer, and Deionized Formamide

10% Ammonium Persulfate

IMPORTANT Use fresh ammonium persulfate. The crystals should crackle as they dissolve.

Step	Action
1	Weigh out 0.50 ± 0.005 g of ammonium persulfate (APS) into a 15-mL polypropylene tube. **! WARNING ! CHEMICAL HAZARD. Always wear appropriate safety attire (full-length laboratory coat, protective glasses, gloves, etc.) when handling and mixing hazardous chemicals. Always work under a chemical fume hood when handling and mixing hazardous chemicals. The room in which you work must have proper ventilation and a waste-collection system.**
2	Using a P-5000 Pipetman or equivalent, add 5 mL of deionized water to the tube.
3	Vortex until all crystals dissolve.

Note Optimally, APS should be prepared fresh daily. At the very least, store 10% stocks at –15 to –25 °C and replace every week. Listen for a crackling sound when adding water to dry APS. Material that has broken down due to high humidity or liquid contamination will no longer crackle when moistened.

10X TBE

To make 500 mL of 10X TBE:

Step	Action
1	To a 500-mL container, add the following: ♦ 54 g Tris ♦ 28 g Boric acid ♦ 4 g Ethylenediaminetetraacetic acid, disodium salt (Na_2EDTA) ♦ Distilled, deionized water to 500 mL **IMPORTANT** Use Tris base (see page 4-2). Use disodium EDTA to make 10X TBE stock. Some major laboratory suppliers provide monosodium EDTA or tetrasodium EDTA.
2	Mix ingredients thoroughly by vortexing.
3	Verify that the pH is 8.2–8.3.

Note 10X TBE stored at room temperature should be used within 1 month. Do not use if a precipitate is present.

Deionized Formamide

IMPORTANT Always use deionized formamide to prepare loading buffers. Over time, formamide hydrolyzes to formic acid and formate. Deionized formamide stock lasts for 3 months at –15 to –25 °C

Step	Action
1	Mix 50 mL of formamide and 5 g of AG501 X8 ion-exchange resin. **! WARNING ! CHEMICAL HAZARD. Formamide is a teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling.**
2	Stir for 30 minutes at room temperature.
3	Check that the pH is greater than 7.0 using pH paper. If the pH is not greater than 7.0, decant the formamide into a beaker containing another 5 g of ion-exchange resin and repeat 30-minute stirring at room temperature.
4	When the pH is greater than 7.0, allow the beads to settle to the bottom of the beaker. Remove the supernatant (formamide), taking care not to disturb the beads.
5	Dispense the deionized formamide into aliquots of 500 µL and store for up to 3 months at –15 to –25 °C.
6	Use one aliquot per set of samples. Discard any unused deionized formamide.

IUB Codes

B

IUB Codes		Complements			
A = adenosine	S = G or C (Strong—3 H bonds)	A	T, U	R	Y
C = cytidine	W = A or T (Weak—2 H bonds)	C	G	Y	R
G = guanosine	Y = C or T (pYrimidine)	G	C	K	M
T = thymidine	B = C, G, or T	T	A	M	K
U = uracil	D = A, G, or T	U	A	S	W
K = G or T (Keto)	H = A, C, or T	D	H	W	S
M = A or C (aMino)	V = A, C, or G	H	D	B	V
R = A or G (puRine)	N = aNy base	N	N	V	B

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C

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WWW Sites

- Bio-Rad Laboratories: http://www.bio-rad.com/cgi-bin/Tango.cgi/ApplicationBits/literature.qry
- Centre National de Séquençage (CNS, or Génoscope): http://www.cns.fr
- Ligochem: http://www.ligochem.com
- Applied Biosystems: www.appliedbiosystems.com/techsupport (Genetic Analysis page: www.appliedbiosystems.com/techsupport)
- QIAGEN: http://www.qiagen.com
- Sanger Centre: http://www.sanger.ac.uk
- The Institute for Genome Research (TIGR): http://www.tigr.org
- University of Oklahoma Advanced Center for Genome Technology (ACGT): http://www.genome.ou.edu
- University of Washington Genome Center: http://www.genome.washington.edu/uwgc
- Washington University School of Medicine Genome Sequencing Center (GSC): http://genome.wustl.edu/gsc

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To Reach Us by E-Mail

Contact technical support by e-mail for help in the following product areas.

For this product area	Use this e-mail address
Chemiluminescence	info@appliedbiosystems.com
Genetic Analysis	galab@appliedbiosystems.com
LC/MS	apisupport@sciex.com
PCR and Sequence Detection	pcrlab@appliedbiosystems.com
Protein Sequencing, Peptide and DNA Synthesis	corelab@appliedbiosystems.com

Regional Offices Sales and Service

If you are outside the United States and Canada, you should contact your local Applied Biosystems service representative.

The Americas	
United States Applied Biosystems 850 Lincoln Centre Drive Foster City, California 94404 Tel: (650) 570-6667 (800) 345-5224 Fax: (650) 572-2743	Latin America (Del.A. Obregon, Mexico) Tel: (305) 670-4350 Fax: (305) 670-4349

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France (Paris) Tel: 33 (0)1 69 59 85 85 Fax: 33 (0)1 69 59 85 00	Portugal (Lisboa) Tel: 351 (0)22 605 33 14 Fax: 351 (0)22 605 33 15
Germany (Weiterstadt) Tel: 49 (0) 6150 101 0 Fax: 49 (0) 6150 101 101	Russia (Moskva) Tel: 7 095 935 8888 Fax: 7 095 564 8787
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Part Numbers

E

ABI PRISM DNA Sequencing Kits and Reagents

Kits

Ready Reaction formulations contain all necessary reagents in one stable premix. Core Kit configurations contain all essential reagents packaged in separate tubes.

ABI PRISM™ dRhodamine Terminator Cycle Sequencing Kits with AmpliTaq® DNA Polymerase, FS

P/N	Kit	Reactions
403044	Ready Reaction	100
403045	Ready Reaction	1000
4303143	Ready Reaction	5000

ABI PRISM BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq DNA Polymerase, FS

P/N	Kit	Reactions
4303573	Ready Reaction	24
4303149	Ready Reaction	100
4303150	Ready Reaction	1000
4303151	Ready Reaction	5000

ABI PRISM BigDye™ Primer Cycle Sequencing Ready Reaction Kits with AmpliTaq DNA Polymerase, FS

P/N	Primer	Reactions
403051	–21 M13	100
403049	–21 M13	5000
403052	M13 Reverse	100
403050	M13 Reverse	5000

ABI PRISM Dye Terminator Cycle Sequencing Kits with AmpliTaq DNA Polymerase, FS

P/N	Kit	Reactions
402080	Ready Reaction	100
402119	Ready Reaction	1000
402118	Core Kit	100

ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kits with AmpliTaq DNA Polymerase, FS

P/N	Primer	Reactions
402111	–21 M13	100
402109	M13 Reverse	100

ABI PRISM Dye Primer Cycle Sequencing Core Kits with AmpliTaq DNA Polymerase, FS

P/N	Primer	Reactions
402071	–21 M13	100
402072	M13 Reverse	100
402073	–21 M13/M13 Reverse	100
402126	T7	100
402127	T3	100
402128	SP6	100
402129	T7/SP6	100
402130	T3/T7	100
402125	Kit reagents only (primerless)	100

Dye-Labeled Primers

Includes 20 pmol of FAM- and JOE-labeled primer, 40 pmol of TAMRA- and ROX-labeled primer, and a control template. Enough for 50 ss- or dsDNA sequencing reactions.

P/N	Primers
401131	–21 M13 Dye Primers (4 x 50), 5´ TGT AAA ACG ACG GCC AGT 3´
401130	M13 Reverse Dye Primers (4 x 50), 5´ CAG GAA ACA GCT ATG ACC 3´
401127	T7 Dye Primers (4 x 50), 5´ TAA TAC GAC TCA CTA TAG GG 3´
401128	T3 Dye Primers (4 x 50), 5´ ATT AAC CCT CAC TAA AGG GA 3´
401129	SP6 Dye Primers (4 x 50), 5´ ATT TAG GTG ACA CTA TAG 3´
403013	PI (+) Dye Primers (4 x 50), 5´ CAG GAC ATT GGA TGC TGA GAA TTC G 3´
403014	PI (–) Dye Primers (4 x 50), 5´ CAG GAG CCG TCT ATC CTG CTT GC 3´

Matrix and Sequencing Standards

P/N	Standard
403047	dRhodamine Matrix Standards Kit
401071	Dye Terminator Matrix Standards Kit
401114	Dye Primer Matrix Standards Kit
4303120	dRhodamine Terminator Cycle Sequencing Standard
4304154	BigDye Terminator Cycle Sequencing Standard
402830	Dye Terminator Cycle Sequencing Standard
401920	Dye Primer Cycle Sequencing Standard

Sample Preparation Reagents

P/N	Standard
402790	ABI PRISM Plasmid Miniprep Kit (100 purifications)
402791	ABI PRISM Plasmid Miniprep Kit (500 purifications)
4305605	5X Sequencing Buffer (600 reactions)
4305603	5X Sequencing Buffer (5400 reactions)

Reagent Kit Protocols

P/N	Protocol
403041	*ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit Protocol*
4303237	*ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit Protocol*
403057	*ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction Kit Protocol*
402078	*ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit Protocol*
402116	*ABI PRISM Dye Terminator Cycle Sequencing Core Kit Protocol*
402113	*ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit Protocol*
402114	*ABI PRISM Dye Primer Cycle Sequencing Core Kit Protocol*
402920	*Primer Island Transposition Kit Protocol*

ABI PRISM 310 Genetic Analyzer

Autosampler Tray Kits

P/N	Kit
402867	48-Tube Sample Tray Kit Includes: 48-Tube Sample Trays (2), 0.5-mL Tube Septa (500), 0.5-mL Sample Tubes (500). (Individual Part Numbers: One 48-Tube Sample Tray, P/N 005572; 0.5-mL Tube Septa, P/N 401956; 0.5-mL Sample Tubes, P/N 401957)
402868	96-Tube Sample Tray Kit Includes: 96-Tube Septa Clips (4), 0.2-mL Tube Septa Strips (24 strips, 480 septa), 0.2-mL Sample Tubes (1000), MicroAmp® Tray and Retainer (10 sets), MicroAmp® Base (10). (Individual Part Numbers: Septa Clips, P/N 402866; 0.2-mL Tube Septa Strips, P/N 402059; 0.2 mL MicroAmp® Tubes, 8/strip, P/N N801-0580; MicroAmp Tray and Retainer, P/N 403081; MicroAmp Base, P/N N801-0531; 96-Well Tray Adapter, P/N 4305051)

Polymers and Consumables

Polymers and Consumables for the ABI PRISM® 310 Genetic Analyzer

P/N	Item	Size/Amt
402837	Performance Optimized Polymer 6 (POP-6™) Generally used for sequencing No template suppression reagent included 200 sample run	3 mL
402844	Performance Optimized Polymer 6 (POP-6) with TSR Includes two 4-mL vials of Template Suppression Reagent 200 sample runs	3 mL
403076	POP-6 polymer with TSR for Shared Instruments Includes eight 4-mL vials of Template Suppression Reagent 200 sample runs	3 mL
402824	10X Genetic Analyzer Buffer with EDTA Used with POP-6 polymer	25 mL
402839	ABI PRISM 310 Capillaries, 47-cm x 50-µm (internally uncoated) Used with POP-6 polymer for rapid sequencing 500 sample runs (100 runs/capillary)	5/pkg
402840	ABI PRISM 310 Capillaries, 61-cm x 50-µm (internally uncoated) Used with POP-6 polymer for long-read sequencing 200 sample runs (100 runs/capillary)	2/pkg
401957	Genetic Analyzer Sample Tubes (0.5-mL)	500/pkg
401956	Genetic Analyzer Septa for 0.5-mL Sample Tubes For 48-Tube Tray	500/pkg
402059	Genetic Analyzer Septa Strips (0.2-mL tube) For 96-Tube Tray	485/pkg (24 strips)

Polymers and Consumables for the ABI PRISM® 310 Genetic Analyzer *(continued)*

P/N	Item	Size/Amt
402866	Genetic Analyzer Retainer Clips (96-Tube Tray Septa Clips)	4/pkg
N801-0580	MicroAmp 0.2-mL Sample Tubes, 8/strip	1000/pkg
403081	MicroAmp Tray and Retainer	10 sets
N801-0531	MicroAmp Base	10/pkg
4305051	96-Well Tray Adapter	1 each
401958	Genetic Analyzer Capillary Cutters	2 each
401955	Genetic Analyzer Buffer Vials (4.0-mL) Includes cap adapters	50/pkg
005914	Platinum cathode electrode	1 each
604418	1.0-mL Glass Syringe Used for GeneScan® and sequencing applications Contains syringe O-rings and ferrule	1 each
604042	GeneScan Glass Syringe (2.5-mL) Contains syringe O-rings and ferrule	1 each
603803	DNA Sequencing Glass Syringe (250-µL) Contains syringe O-rings and ferrule	1 each
221102	Syringe O-rings O-ring inside of glass syringe assembly	1 each
005401	Syringe ferrule Ferrule inside of glass syringe assembly	1 each
005404	Capillary Fitting Screw fitting used to hold the capillary in the pump block	1 each
005572	0.5-mL Sample Tray Holds 48 0.5-mL sample tubes	1 each
603796	Waste vial Vial attaches to the gel pump block, collects waste generated during gel pump priming with Sequence Polymer	1 each
005402	Anode buffer jar Buffer jar attaches to gel pump block, holds the anode buffer	1 each
604076	Valve, waste vial Gel pump block manual valve, the waste vial attaches to the fitting on this valve	1 each
604075	Valve, plastic syringe, Luer Gel pump block manual valve, the DNA sequencing polymer plastic syringe attaches to the fitting on this valve	1 each
310021	Thermal Tape For affixing the capillary to the heat plate	1 each

Chemical Installation Kits

These kits are shipped with new instruments for the purpose of installation and training.

P/N	Kit
402089	ABI PRISM 310 Basic Install Kit Included with purchase of ABI PRISM 310 Genetic Analyzer Includes: 310 Genetic Analyzer Buffer with EDTA, Leak Test Capillary, Sensitivity Standard, Genetic Analyzer Buffer Vials, Genetic Analyzer Septa, Genetic Analyzer Capillary Cutters, 5-mL Syringe
402090	ABI PRISM 310 DNA Sequencing and GeneScan Install Kit Included when both the DNA Sequencing Analysis and GeneScan Modules (677-30 and 672-30, respectively) are purchased Includes: POP-6 polymer, TSR, 61-cm x 50-µm i.d. Capillaries, POP-4™ polymer, 47-cm x 50-µm i.d. Capillaries, 310 Genetic Analyzer Buffer with EDTA, Fluorescent Genotyping Demonstration Kit B, GeneScan-500 [TAMRA] Internal Lane Size Standard, formamide, Amberlite MB-1A, Dye Primer Matrix Standards, Dye Terminator Matrix Standards, Fluorescent Amidite Matrix Standards, NED Matrix Standard, dRhodamine Matrix Standards, BigDye Terminator Cycle Sequencing Standard
401820	ABI PRISM 310 DNA Sequencing Install Kit Included with purchase of the DNA Sequencing Analysis Module (677-30) Includes: POP-6 polymer, TSR, 61-cm x 50-µm i.d. Capillaries, dRhodamine Matrix Standards, BigDye Terminator Cycle Sequencing Standard

ABI PRISM 377 DNA Sequencer

Plates and Spacers

P/N	Item
401878	48-cm Glass Plates/Spacers Kit Includes: two sets of 48-cm Well-to-Read Glass Plates and Gel Spacers
4305810	48-cm Glass Plates/Spacers Kit for the for the ABI PRISM® 377 with 96-Lane Upgrade Includes: one set of 48-cm Well-to-Read Glass Plates and Gel Spacers
401876	36-cm Glass Plates/Spacers Kit Includes: two sets of 36-cm Well-to-Read Glass Plates and Gel Spacers
4305693	36-cm Glass Plates/Spacers Kit for the ABI PRISM 377 with 96-Lane Upgrade Includes: one set of 36-cm Well-to-Read Glass Plates and Gel Spacers
401835	48-cm Rear Glass Plate
401838	48-cm Front Glass Plate
4305387	48-cm 0.4-mm Stepped Front Glass Plate for the ABI PRISM 377 with 96-Lane Upgrade
401837	Two 48-cm Gel Spacers, 0.2 mm thick
401839	36-cm Rear Glass Plate
401840	36-cm Front Glass Plate
4305384	36-cm 0.4-mm Stepped Front Glass Plate for the ABI PRISM 377 with 96-Lane Upgrade
401836	Two 36-cm Gel Spacers, 0.2 mm thick

Combs

P/N	Item
402168	18-well Sharktooth Comb, 0.2 mm thick
401827	24-well Sharktooth Comb, 0.2 mm thick
401922	32-well Sharktooth Comb, 0.2 mm thick
401828	36-well Sharktooth Comb, 0.2 mm thick
402177	48-well Sharktooth Comb, 0.2 mm thick
402180	64-well Sharktooth Comb, 0.2 mm thick
4305385	100-well Sharktooth Comb, 0.4 mm thick for the ABI PRISM 377 with 96-Lane Upgrade

Cassette, Buffer Chambers, and Heat Plate

P/N	Item
604297	Gel Cassette
401991	Gel Pouring Fixture Kit (contains top and bottom fixtures, clamps, and syringe)
4304406	Upper Buffer Chamber- new
4304409	Gasket Replacement Kit for Upper Buffer Chamber P/N 4304406
604078	Upper Buffer Chamber- obsolete
604524	Gasket Replacement Kit for Upper Buffer Chamber P/N 604078
603875	Lower Buffer Chamber
603822	Upper Buffer Electrode Assembly
603823	Lower Buffer Electrode Assembly
603833	Front 36-cm Well-to-Read Heat Plate

ABI 373 DNA Sequencer

Combs and Spacers

P/N	Item
401472	24-well Sharktooth Comb, 0.4 mm thick
401580	32-well Sharktooth Comb, 0.4 mm thick
401473	36-well Sharktooth Comb, 0.4 mm thick
402179	48-well Sharktooth Comb, 0.4 mm thick
402182	64-well Sharktooth Comb, 0.4 mm thick
402214	Casting Comb for Sharktooth, 0.4 mm thick
402048	Uni-length Glass Plate Spacer, 0.4 mm thick
401582	24-well Sharktooth Comb, 0.3 mm thick
401581	32-well Sharktooth Comb, 0.3 mm thick
401583	36-well Sharktooth Comb, 0.3 mm thick
402178	48-well Sharktooth Comb, 0.3 mm thick
402181	64-well Sharktooth Comb, 0.3 mm thick
402215	Casting Comb for Sharktooth, 0.3 mm thick
402047	Uni-length Glass Plate Spacer, 0.3 mm thick

Plates

P/N	Plate
401617	48-cm Notched Glass Plate (Stretch Configuration)
401618	48-cm Plain Glass Plate (Stretch Configuration)
401069	24/34-cm Notched Glass Plate (Stretch Configuration)
401498	24/34-cm Plain Glass Plate (Stretch Configuration)

Buffer Chambers

P/N	Item
603750	Small Upper Buffer Assembly
603751	Large Upper Buffer Assembly
603410	Lower Buffer Assembly
200576	Gasket (Silicone Foam Cord) for Upper Buffer Chamber Seal
201410	Gasket Adhesive (for use with P/N 200576)

Documentation and Software

User's Manuals and Other Documents

P/N	Item
903565	*ABI PRISM 310 Genetic Analyzer User's Manual*
902376	*373 DNA Sequencing System User's Manual*
904258	*373 DNA Sequencer With XL Upgrade User's Manual*
4304367	*Using the ABI 373 BigDye Filter Wheel User Bulletin*
903433	*ABI PRISM 377 DNA Sequencer User's Manual*
904210	*ABI PRISM 377-18 DNA Sequencer User's Manual*
904412	*ABI PRISM 377 DNA Sequencer XL Upgrade User's Manual*
4305423	*ABI PRISM 377 DNA Sequencer 96-Lane Upgrade User's Manual*
903939	*CATALYST 800 Molecular Biology LabStation User's Manual*
904414	*ABI PRISM 877 Integrated Thermal Cycler User's Manual*
904532	*ABI PRISM DNA Sequencing Analysis Software (Version 3.0) User's Manual*
4304075	*ABI PRISM DNA Sequencing Analysis Software (Version 3.2) User's Manual*
770901	*Comparative PCR Sequencing, A Guide to Sequencing-Based Mutation Detection*
770905	*Guide to PCR Enzymes*
4304655	*Precipitation Methods to Remove Residual Dye Terminators from Sequencing Reactions User Bulletin*

Software

P/N	Item
4303032	ABI PRISM 310 Training CD
402089	Primer Express™ Software

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